Supplementation of *Cannabis sativa* L. leaf powder accelerates functional recovery and ameliorates haemoglobin level following an induced injury to sciatic nerve in mouse model

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Abstract: Peripheral nerve injury is a common condition with a multitude of signs and symptoms. The major consequence of injury is limited physical activity. Presently, we are lacking effective therapies for PNI and it is need of the hour is to explore potential remedies for the recovery of functional loss. Here, we have investigated the role of crude *Cannabis sativa* L. leaf powder in promoting functions recovery, in mouse model subjected to a traumatic sciatic nerve injury. A dose of 200mg/kg of the body weight per day was administered orally from the day of nerve crush till the end of the experiment. The motor functions were evaluated by measuring sciatic functional index, muscle grip strength and muscle mass; whereas the sensory functions were assessed by hotplate test. The haematology and serum analyses were carried out to estimate the effect of treatment on the systemic index and oxidative stress. The gain of motor functions was significantly improved and was early noticed in the treated mice. Restoration of muscle mass and elevated haemoglobin level were statistically significant in the treatment group. This study indicates that *Cannabis sativa* L. supplementation accelerates the motor functions recovery after nerve compression injury.

Keywords: Peripheral nerve injury, functional recovery, *Cannabis sativa* L., oxidative stress, haemoglobin.

INTRODUCTION

Peripheral nerve injury (PNI) is a common condition with a multitude of signs and symptoms depending on the nerves involved and severity of injury. It can be caused by sharp lacerations, road accidents, gunshot wounds, and trauma. It interrupts the normal functioning of the motor and sensory neurons by causing demyelination (Alvites *et al.*, 2018). It may result in the development of muscular atrophy and loss of neuronal function. The nervous system is the most complicated system in living organisms, so the functional recovery is either incomplete or slow. This ultimately leads to muscular atrophy (Tuffaha *et al.*, 2016). Thus, the need of the hour is to explore natural products to accelerate the recovery process, before the onset of muscular atrophy. Unfortunately, there is a great dearth of potent therapeutics or remedies to combat an injury to CNS/PNS.

Plants derived bioactive compounds commonly known as phytochemicals, are diverse in nature. A large number of natural compounds have been proposed for a general use, keeping in view their multitudinous health related benefits. They have anti-oxidative, anti-inflammatory, analgesic, anti-diabetic, anti-microbial and various other properties (Pant *et al.*, 2017). They have a reduced risk of side effects and can be a good alternative to the synthetic medicines to cure the diseases. Among the innumerable classes of the phytochemicals, several have been discovered and demonstrated to exert neuroprotective effects (Hussain *et al.*, 2018; Hussain *et al.*, 2018).

Cannabis sativa L. (*C. sativa* L.) is a yearly herbaceous flowering plant native to the eastern Asia and belongs to the family Cannabaceae. It is commonly known as marijuana or hemp. It naturally occurs throughout the planet, in several humid and tropical parts. It possesses anti-inflammatory, analgesic, sedative, anti-convulsive, and hallucinogenic properties (Andre *et al*., 2016). Its principal constituents are tetrahydrocannabinol (THC) and cannabidiol (CBD) that have been investigated for

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their potential pharmacological effects such as antitumor, antipsychotic, anti-anxiety, immunomodulatory, neuroprotective, anti-oxidative and anti-inflammatory effects (Andre *et al*., 2016). It has been reported that THC, CBD and some other extracts of *C. sativa* L. exhibit anti-inflammatory, anti-nociceptive properties against neuropathic pain (Larsson and Lagerås, 2015). A combination of THC and CBD reduces the neuro-inflammation induced by the amyloid beta (Aβ) peptides in Alzheimer's disease (AD) (Aso *et al.*, 2015). The reported data on *C. sativa* L. make it a potential choice for assessing its role in the improvement of PNI sequelae. Subsequently, there is a great dearth of information regarding the capability of *C. sativa* L. to promote peripheral nerve recovery. Thus, we took the initial step to explore the possible effects of *C. sativa* L. in terms of promoting functional retrieval after peripheral nerve injury.

MATERIALS AND METHODS

Animals

The mice were procured from the animal facility of Department of Physiology, Government College University Faisalabad-Pakistan. A total of 14 albino male mice weighing approximately 32g with an average age of 6-7 weeks, were kept in the animal housing facility at 23±3℃ and were exposed to a 12 hours' light/dark cycle. Mice were regularly given rodent chow diet and water *ad libitum*.

Sciatic nerve compression injury

For the induction of peripheral nerve injury, mice were anesthetized by injecting a mixture of xylazine (5mg/kg body weight) and ketamine (70mg/Kg body weight) intraperitoneally. The process of nerve crush was done on the right hind limb while the left one was considered as control. The incision region was smoothly shaved, and the skin cut over a measurement of 2cm along the proximal half of the mark b/w the knee joint and the trochanter major. The sciatic nerve injury was induced on right limb by applying a constant pressure for 15 seconds by using a pair of forceps. The nerve compression was ensured that the nerve was perfectly crushed and the epineurium had remained intact. This was achieved by slightly raising the nerve with a microprobe to observe the clear site in the nerve, which indicated a complete nerve press. The incisions were sutured by 4-0 stitches and mice were placed on the hot pad (Hussain *et al.*, 2013; Ramli *et al.*, 2017). After inducing injury in all mice, they were divided into two groups; treatment group (n=7) and control group (n=7).

Plant supplementation

The leaves were shade dried and ground into course powder. A measured quantity of crude *C. sativa* L. leaves powder at a dose of 200mg/kg body weight was mixed in rodent chow diet. It was ensured that average daily consumed diet of 5gram contained required dose of plant material. The dose containing diet was administered to the experimental group from the day of nerve crush till the end of the experiment. The average food consumption and body weight were measured daily.

Behavioral tests

Sciatic functional index (SFI)

The SFI was employed to evaluate the motor functions recovery. The hind paws were painted by using ink and the mouse was allowed to walk on a 50cm long and narrow wooden track with white paper on the floor. All the measurements from the most clearly inked prints per run were selected from experimental (E) and normal (N) side and the following formula was used for the calculation of SFI:

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

The distance from heel to tip of the third toe is print length (PL), the distance between second to the fourth toe is intermediary toe spread (IT), and the distance between first and fifth toe is toe spread (TS). NIT, NTS, and NPL is the IT, TS and PL of contralateral (normal) paws whereas EIT, ETS and EPL is the IT, TS and PL of ipsilateral (experimental) paws (Ma *et al.*, 2016).

Muscle grip strength

It is a non-intrusive procedure, used for evaluating the strength of mouse' muscle *in vivo* by using a horizontal grid or metal bar (Bioseb, Chaville, France). The mouse was positioned over a metallic grid and was allowed to grab to thwart the unintentional backward movement carried out by the experimenter until the pulling force overcomes grip strength of animal. The peak pull force was marked by strength-meter when the mouse lost its grip. Strength was evaluated for the both hind limbs; contralateral and ipsilateral. A mean of 3 readings was taken for each mouse and the readings of experimental group were compared to the normal group to ensure functional recovery (Hussain *et al*., 2013).

Hot plate test

The sensory functions recovery was evaluated by performing a hot plate test (SCILOGEX MS7-H550-S LED digital 7x7 Hotplate stirrer). Before the actual test, the mouse was acclimatized to the non-operative hot plate for a minute. The individual mouse was placed to stand in such a position that its operated paw was on the hot surface of plate set at a temperature of 56±2°C until the mouse jumped or licked its hind paw. This value was noted as hot plate latency (HPL). The mouse was immediately removed from the surface after such

reaction. The latency period was noted by the use of stopwatch measured as withdrawal reflex (WRL). The 3 readings were noted with an interval of 2 minutes between the successive readings. If the mouse does not show any response for 30 seconds, the thermal stimulus would be stopped to prevent tissue injury and the WRL was noted as 30 seconds (Mene *et al*., 2002; Imam and Sumi, 2014).

Biochemical tests

Total antioxidant capacity (TAC)

The chief function of antioxidants is to provide defense against the damaging effects of free radicals. Total antioxidant capacity is used to evaluate the antioxidant status of a biological sample and can assess the antioxidant reaction against the free radical species (Rubio *et al.*, 2016a). Trolox equivalent antioxidant capacity or TEAC is the most common direct assay.

a) Principle

The TAC assay is based on the principle that when 2, 2´-azinobis 3 ethylbenzothiazoline-6-sulfonate (ABTS) is incubated with H_2O_2, a radical of ABTS (ABTS•+) is formed. The ABTS•+ is blue-green in color and has the maximum absorption at 650nm, 734nm and 820nm. The antioxidants present in the sample decrease the ABTS•+ and suppress the color production which is inversely proportional to the total antioxidant capacity of the serum sample. The rate of reaction is calibrated with the Trolox which is a water-soluble equivalent of vitamin-E and the results are measured as mmol Trolox equivalent/L (Rubio *et al.*, 2016b).

b) Reagents

Acetate buffer (Reagent 1)

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

ABTS (Reagent 2)

A solution of acetate buffer (30mM/L) having a pH of 3.6 and solution of glacial acetic acid was prepared in deionized water. The solutions were mixed in such a way that a final pH of 3.6 was achieved. The above mixture was diluted with the solution of commercial H_2O_2 and the final concentration was 2mM/L. ABTS (0.549g) was dissolved in 100ml of the prepared buffer solution. The resultant concentration of this ABTS in buffer was 10mM/L. The solution was incubated at room temperature for 1 hour. The solution was incubated at room temperature for at least one hour and stored at the temperature of 4ºC. At room temperature, the stability of this solution is 6 months.

c) Procedure

For the spectrophotometric analysis, semi-auto chemistry analyzer (Biosystem, BTS-330) was used. The monochromatic wavelength of 650nm was adjusted and a 5 minutes' time period was provided to the filter to get warm. The reagent 1 (200ul) was pumped and considered as blank. 5µl sample was mixed with the reagent 1 (200µl). After that, 20µl of reagent 2 was added and absorbance was measured after the incubation of 5 minutes at room temperature. By the calibration of standards of vitamin C, the results were calculated. The linear type of calibration was used.

Total oxidant status (TOS)

Oxidative stress is reported to be one of the reasons of the neural damage after PNI. Total oxidant status is usually used to evaluate the overall oxidative status of the body (Wu *et al*., 2017).

a) Principle

Due to oxidants presence in the sample, the oxidation of complex of *o*-dianisidine ferrous ion into ferric ion occurs. Then xylenol orange makes a complex of color by reacting with the ferric ions in an acidic environment. The intensity of the color is related to the degree of oxidant molecules that persist in the biological samples and it can be evaluated spectrophotometrically. The hydrogen peroxide (H_2O_2) is used for the calibration. The results are expressed as µmol H_2O_2 Equivalent/L (Motor *et al.*, 2014).

b) Reagents

Reagent 1

In this step, 114 grams of xylenol orange were dissolved with NaCl (8.18 grams) in 900ml sulphuric acid. Glycerol (100ml) was then added to make it 1000ml as final volume. The resultant solution comprised of 140mM NaCl, 150 µM xylenol orange, and 1.35 M glycerol. The final pH should be 1.75 and its stability is 6 months at the temperature of 4ºC.

Reagent 2

In this step, 3.17 grams of *o*-dianisidine-dihydrochloride were dissolved with 1.96 grams of ferrous ammonium sulfate in thousand ml of sulphuric acid (25mM). The solution comprised of *O*-dianisidine-dihydrochloride (10mM) and ferrous ammonium sulfate (5mM). Its stability is 6 months at the temperature of 4ºC.

c) Procedure

The reagent 1 (225µL) was mixed with 35µl of serum sample. The initial absorbance was taken after mixing them. Then reagent 2 was mixed (11µL) with the mixture of sample and reagent 1. The mixture was incubated for 4mins at room temperature. Absorbance was taken at bichromatic 560 nm wavelength by using spectrophotometer (Biosystem, BTS-330).

Muscle weight

The muscles were weighed to estimate the extent of muscular atrophy. The Tibialis anterior (TA) and gastrocnemius (Gastroc) muscles of both normal chow and cannabis chow groups were used for mass evaluation. The weights of both groups (ipsilateral legs) were compared by the termination of the experiment to find the substantial difference between the muscle mass of untreated and treated mice.

Ethical approval

The project was approved by the Animals Experiment Ethical Committee of Government College University, Faisalabad- Pakistan. All the experiments were carried out in accordance with relevant regulations and guidelines.

RESULTS

Effects of Cannabis sativa* L. *treatment on food consumption and body mass

We measured the food consumption during the entire period of the experiment (before and after the sciatic nerve lesion). It was found that the food consumption in both normal chow and the cannabis chow mice groups remained unaffected (fig. 1A). The taste of *C.sativa* L. in the treatment group did not alter the concentration of food consumption. It means that whatever results we obtained; either negative or positive; were exclusively due to the addition of *C.sativa* L. in the diet of treatment group. The body weight of both normal chow and the cannabis chow mice groups was almost equivalent before and after the sciatic nerve lesion (fig. 1B). It indicates that *C.sativa* L. did not affect the body mass. Moreover, the body mass and the food consumption also remained unaffected even in stressful circumstances that were observed after nerve lesion.

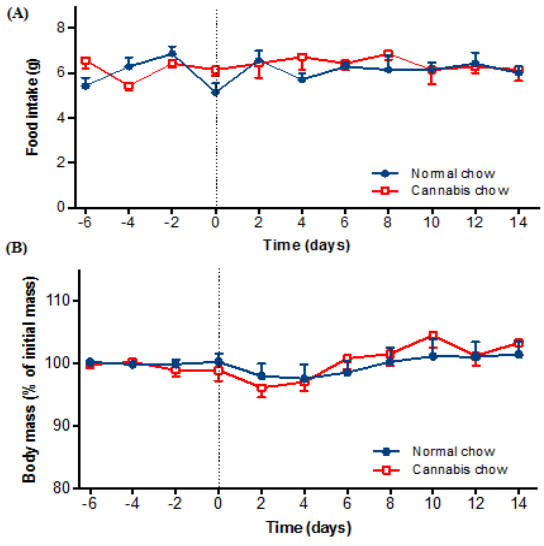

Fig. 1: Cannabis-containing diet does not alter food intake neither body mass after nerve injury. (A) Time course of food intake in mice fed on normal chow (blue circles, n=7) or cannabis chow (red squares, n=7). Mice were fed on cannabis chow at the time of sciatic nerve crush (dotted line at day 0) and during the whole period of functional recovery. Two-way repeated-measure ANOVA (diet x time) showed non-significant effects of diet ($F_{(1,12)}$=1.15, P=0.304) and time ($F_{(10,120)}$=1.16, P=0.304), and a non-significant interaction between factors ($F_{(10,120)}$=1.34, P=0.215). (B) Time course of body mass of mice as in A. Body mass is expressed as a percentage of initial mass at days -6 and -4 per individual. Two-way repeated-measure ANOVA (diet x time) showed a significant effect of time ($F_{(10,120)}$=4.99, P=0.0001), a non-significant effect of diet ($F_{(1,12)}$=0.04, P=0.836) and a non-significant interaction between factors ($F_{(10,120)}$=1.03, P=0.425).

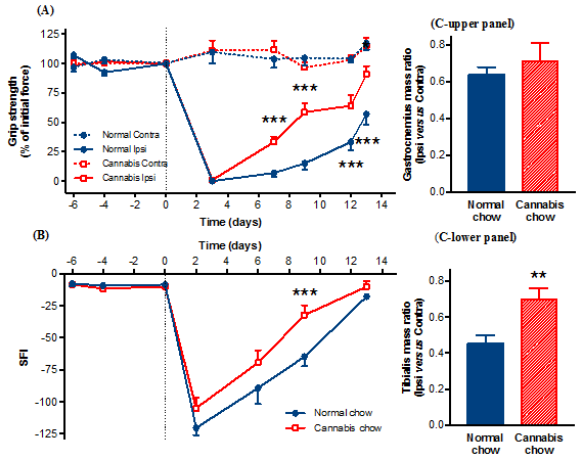

Fig. 2: Cannabis-containing diet accelerates motor function recovery after nerve injury. (A) Time course of muscle grip strength in mice fed on normal chow (blue circles, n=7) or cannabis chow (red squares, n=7). Mice were fed on cannabis chow at the time of sciatic nerve crush (dotted line at day 0 and during the whole period of functional recovery). Measurements were obtained from hind limbs contralateral (dotted lines) and ipsilateral (solid lines) to the lesion. Grip strength is expressed as a percentage of initial force developed at days -6 and -4 per individual. Two-way repeated-measure ANOVA (diet x time) showed significant effects of diet ($F_{(1,12)}$=19.9, P=0.0008) and time ($F_{(7,84)}$=140.8, P<0.0001), and a significant interaction between factors ($F_{(7,84)}$=8.31, P<0.0001). Post-hoc pairwise comparisons with Benjamini-Hochberg correction revealed significant differences between normal and cannabis chow at 7, 9 12 and 13 days after lesion (***P<0.001). (B) Time course of sciatic functional index (SFI) of mice as in A. Two-way repeated-measure ANOVA (diet x time) showed a significant effect of time ($F_{(6,72)}$=127.1, P=0.0001) and a significant interaction between factors ($F_{(6,72)}$=3.18, P=0.0079). The effect of di*et al*most attained significance ($F_{(1,12)}$=4.04, P=0.067). Post-hoc pairwise comparisons with Benjamini-Hochberg correction revealed a significant difference between normal and cannabis chow at 9 days after lesion (***P<0.001). (C) Gastrocnemius

(upper panel) and Tibialis (lower panel) muscle mass in mice as in A. Measurements are expressed as a ratio between hind limbs ipsilateral and contralateral to the lesion. Unpaired t-test showed a significant effect of diet in tibialis muscle (**P<0.01).

Cannabis sativa L. *accelerates motor functional recovery*

The hallmark of the sciatic nerve injury is the complete functional loss of motor neurons. The progression of functional retrieval is directly associated with the nerve regeneration rate which is extremely slow under ordinary situations. Before the complete functional retrieval, the conditions become worse due to the aggravation of muscular atrophy. So, the need of hour is to accelerate the rate of nerve regeneration and functional retrieval before the occurrence of muscular atrophy. The functional recovery in the mouse model of the mechanically crushed sciatic nerve was evaluated by measurement of the sciatic functional index (SFI), grip strength of muscles, and muscular weight. The addition of *C. sativa* L. restored motor function more rapidly in cannabis chow group as compared to the normal chow group. The grip strength of muscle improved significantly in cannabis chow group (fig. 2A). The pattern of recovery in the measurement of SFI was similar to that of the grip strength retrieval in the cannabis chow group (fig. 2B). The measurement of muscular weight is also another reliable parameter which supports the occurrence of functional retrieval. The TA muscle assists in running, walking, hiking, and all other activities which involve the movement of the leg. The Gastroc muscle is a big muscle and forms a part of the calf muscle and is involved in hind limb movement. So, we measured and compared the masses of the TA and Gastroc as they can act as a suitable reference for the retrieval process. The muscle mass of the TA muscle was significantly increased, almost close to the contralateral hind limb in the cannabis chow group (fig. 2C – lower panel). These results lend credence to the idea that *C. sativa* L. in crude form can accelerate functional retrieval and can delay the occurrence of muscle atrophy. Moreover, the pattern of mass recovery was also seen in the Gastroc muscle of cannabis chow group, but the data was not statistically significant statistically (fig. 2C – upper panel).

Cannabis sativa L. *accelerates sensory functional recovery*

Both sensory and motor neuron functionality is disturbed in case of sciatic nerve injury. Thus, the trend of functional recovery after nerve crush is also indicated by the functional recovery of sensory neurons. In our study, the thermal sensation recovery was evaluated by the hot plate test. Although the data was not significant statistically, but we noticed that there was a pattern of decreased withdrawal latency of ipsilateral hind paw in mice of cannabis chow group (fig. 3).

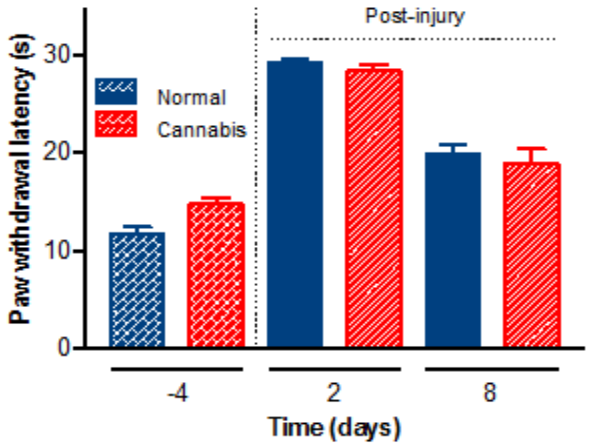

Fig. 3: Cannabis-containing diet does not modify detection of pain after nerve injury. Paw withdrawal latency in response to thermal stimulation in mice fed on normal chow (blue columns, n=7) or cannabis chow (red columns, n=7). Measurements were obtained before and after sciatic nerve crush and subsequent functional recovery. Two-way repeated-measure ANOVA (diet x time) showed a significant effect of time ($F_{(2,24)}$=174.6, P<0.0001), a non-significant effect of diet ($F_{(1,12)}$=0.4, P=0.539) and a significant interaction between factors ($F_{(2,24)}$=3.69, P=0.04). Post-hoc pairwise comparisons with Benjamini-Hochberg correction did not reveal any significant differences between normal and cannabis chow.

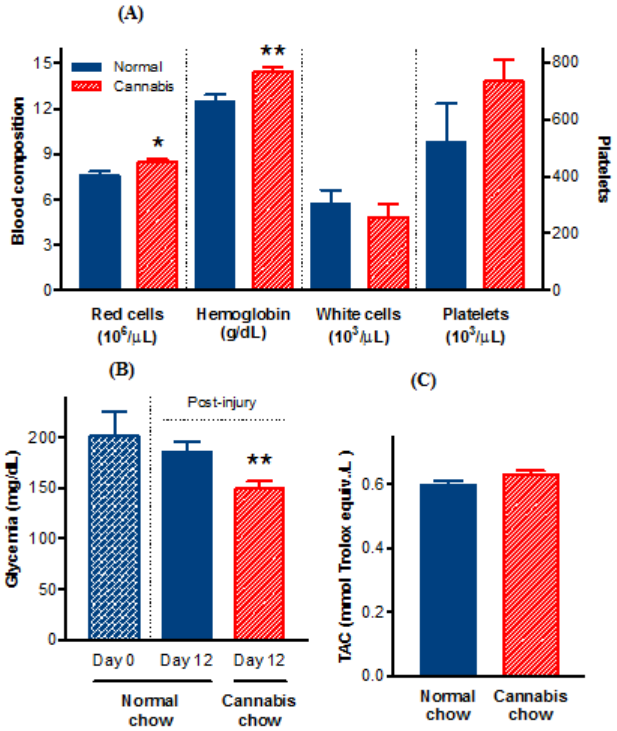

Fig. 4: Effect of cannabis-containing diet on systemic indexes. (A) Blood cell count and haemoglobin content in mice fed on normal chow (blue solid columns, n=7) or

cannabis chow (red hatched columns, n=7). Measurements were obtained after sciatic nerve crush and subsequent functional recovery. Unpaired t-test on each parameter showed a significant effect of diet (**P*<0.05; ***P*<0.01). (B) Glycaemia measured in mice as in A at the time of sciatic nerve crush (day 0, blue hatched column, n=7); and after 12 days of functional recovery subsequent to sciatic nerve crush in mice fed on normal chow (blue solid column, n=7); or cannabis chow (red hatched column, n=7). Unpaired t-test on post-injury measurements showed a significant effect of diet (***P*<0.01). (C) Total anti-oxidant capacity (TAC) of mice as in A. Unpaired t-test did not show any significant difference.

Impacts of Cannabis sativa* L. *Treatment on the Systemic Indexes

There were somewhat variable results of haematological analyses of both normal chow and cannabis chow mice groups. The red blood cells (RBCs) are formed by hormone erythropoietin and the erythropoietin accelerates the motor functions of the sciatic nerve. Moreover, platelets also promote the occurrence of peripheral nerve regeneration. To know whether *C. sativa* L. exerts any effect on haematology, we evaluated the white blood cells (WBCs), RBCs and platelets count and haemoglobin level. In the cannabis chow group, the RBCs count, and haemoglobin level were augmented significantly, but the WBC and platelets counts were statistically non-significant (fig. 4A). There is no previous report regarding the anti-diabetic efficacy of *C. sativa* L., so we measured the glucose level in both normal chow and cannabis chow mice groups to find out whether *C. sativa* L. exerts anti-diabetic effect or not. The results were statistically significant regarding glucose lowering capability of *C. sativa* L. (fig. 4B). The present finding merits further assessment to elucidate any potential anti-diabetic role of *C. sativa* L. There was also a noticeable augmented TAC in the mice of cannabis chow group but the data was not significant statistically (fig. 4C).

DISCUSSION

To date, there is no report regarding the effects of *C. sativa* L. on peripheral nerve lesion. The present study was designed to find out the potential role of crude *C. sativa* L. in accelerating the peripheral nerve regeneration. Our findings indicate a rapid recovery of motor functions in *C. sativa* L. treated group. The muscle grip strength was significantly ameliorated and was noticeable even at day 7, whereas SFI measurement showed an improvement in motor functions on day 9 in the treated group. The PNI leads to muscular atrophy and results in loss of muscular weight (Tuffaha *et al.*, 2016). It is considered proportional to the degree of innervation, as the denervated muscles endures progressive atrophy and thus considered as a parameter for functional recovery (Li *et al.*, 2013; Navarro, 2016). An increase in the muscle mass was another indication of ameliorating effects offered by *C. sativa* L. The weight of TA and Gastroc muscles was measured after decapitation. The muscular mass of both TA and Gastroc muscles appeared almost normal in the treated group at the termination of the experiment. In the treated group, the mass of TA and Gastroc muscles of ipsilateral limbs was nearly close to the contralateral hind limb. This proposes that *C. sativa* L. has the ability to restore the nerve functions before the initiation of muscular atrophy. The results of the present study show the therapeutic efficiency of *C. sativa* L. as a neuroprotective agent to accelerate the peripheral nerve regeneration. Oxidative stress causes neuronal damage by initiating mitochondrial dysfunction, demyelination, neuro-inflammation, and apoptosis while the reduced oxidative stress or enhanced antioxidants improves the functional recovery after peripheral nerve injury (Areti *et al.*, 2014). It has been reported that *C. sativa* L. possess anti-oxidative and anti-apoptotic properties (Iuvone *et al.*, 2004; Lee *et al.*, 2017). We observed a similar trend in the treated group, although the difference between anti-oxidant status of both treated and untreated groups was not statistically significant.

Since the nature of sciatic nerve is of mixed type, therefore, we also analyzed the restoration of sensory functions to evaluate the functional retrieval. We found that there was a noticeable decrease in withdrawal latency of ipsilateral hind paw of the treated group animal, but the data was not statistically significant. This outcome highlights the protective effect of *C. sativa* L. and encourages further evaluation. Furthermore, erythropoietin promotes the RBCs formation and the administration of erythropoietin accelerates the motor functions recovery in sciatic nerve injury model (Chan *et al.*, 2014; Sundem *et al.*, 2016). We found that the haemoglobin level and RBC count were significantly increased in cannabis treated group as compared to the control group. This opens a new avenue and indicates that the functional recovery may be due to the enhanced erythropoietin level elicited by the administration of *C. sativa* L. In conclusion, crude leaf powder of *C. sativa* L. accelerates the motor functions recovery and regeneration of the peripheral nerve. Moreover, there is a need to investigate the response of higher dose and bioactive compounds of *C. sativa* L. that are responsible for these health-promoting effects. It will be interesting to investigate the effect of other parts of this plant in the context of promoting peripheral nerve regeneration. Moreover, it will be interesting to explore the molecular mechanisms and nerve and muscle fibers morphology.

CONCLUSION

In the nut shell, findings of this study prove that *C. sativa* L. exhibits a potential to accelerate the functions recovery

after a compression injury to Sciatic nerve. Although, these results are very promising but further detailed investigations are suggested to explore the effective constituents that are actual players of promoted recovery process. In future, *C. sativa* L. can prove a novel therapeutic agent for the peripheral nerve regeneration as observed in traumatic injury cases.

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