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Indian ginseng (N.Hexane and Chloroform extracts) offers ameliorating effects on muscle functions restoration in a mouse model of peripheral nerve injury

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Abstract

Peripheral nerve injuries fall among most commonly happening medical situations nowadays. Such injuries occur in response to gunshots, road traffic accidents, mechanical crushes and traumas which eventually result into compromised sensory and motor activities. Despite having wonderful advancements in pharmacological therapies, such injuries are still waiting for their first line treatment. The present study is planned to assess the effect of n.Hexane and Chloroform extract of Indian ginseng root on sensorimotor function revival in mice induced with sciatic nerve crush lesion. Twelve male BALB/C mice were divided into control and 2 treatment groups having an equal number of mice (n=4) per group. Control was offered routine diet, while treatment groups (n.Hexane and Chloroform) were offered diet mixed with their respective treatments (100mg/kg daily dose of each extract of Indian ginseng root) from the day of the nerve crush and then until the end of the trial. Motor and sensory functions retrieval was assessed through sciatic functional index, grip strength, and hot plate tests which were found to be highly significant for Chloroform treated group. This group also showed prominent increase in skeletal muscle mass. The significant reduced (p=0.01) total oxidant status and increased total antioxidant capacity (p=0.01) in this group suggest that Indian ginseng root's Chloroform extract has prominent potential for ameliorating the muscle function restoration as compared to control and n-Hexane group.

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Introduction

Peripheral nerve injuries (PNIs) are the most abundant type of health ailment which results in the limited activity of affected individuals (Maqbool *et al.,* 2020). The lessened nerve becomes unable to innervate the target segment of the body and hence results in the decreased quality of life due to limited physical activity with immense cost (Li *et al.,* 2014).

Peripheral nerves are long distant bundle of axons that are assigned to coordinate central nervous system to the whole of the body. Due to delicate thread like structure without having tough covering, nerves are more prone to be injured as a result of any kind of traumatic stimulus (Kamble *et al.,* 2019). Luckily, peripheral nerves possess innate ability to regenerate once they get injury, but the process of nerve regeneration is as slow as it takes months or even years to accomplish (Osborne *et al.,* 2018). Eventually, the target tissue (organ or muscle) starts atrophying (an irreparable loss) due to lack of innervation for an unlimited time.

To speed up this process of regeneration in order to avoid the irreversible loss of target tissue require therapeutic strategies (Faroni *et al.,* 2015). Most of therapeutic strategies regarding the PNIs management have been adopted yet they all have some limitations. Therefore, in spite of having a plenty of advancements made in medicine, such injuries are still waiting for their first line therapy (Gordon & English, 2016).

The nature is full of plants having a rich source of therapeutically potent compounds labeled as phytochemicals which can be used to cure different diseases (Manjoosha *et al.*, 2010). Phytochemicals comprise of complex mixture of organic compounds such as terpenes, saponins, tannins, glycosides, flavonoids, alkaloids, and fatty acids (Kumar & Khanum, 2012) and have been known to have various therapeutic properties. That is the reason that risk of many diseases may be reduced by the regular consumption of vegetables and fruits (Zhu *et al.*, 2018). In addition, plant derived drugs exist as an imperious part of modern medicine which have been discovered by keeping in mind the historic use of their source plant by their local population as remedies against various diseases (Pandey *et al.*, 2013).

Indian ginseng (*Withania somnifera*) is one of these medicinaly important plants. It belongs to the Solanaceae/nightshade family and is also known as poison gooseberry and ashwagandha. In Ayurveda system of medicine, this plant has been reported to be used for anxiety, hepatotoxicity, hyperlipidemia, inflammation (Kalra & Kaushik, 2017), Parkinson's disease (Khuwaja *et al.,* 2011) and other neurological disorders (Kuboyama *et al.,* 2005). Individual components and extracts of Indian ginseng have portrayed therapeutically effective results in numerous models of neurological disorders like Alzheihmer's Disease, Huntington's Disease, Parkinson's Disease, stress and epilepsy as well (Kulkarni & Dhir, 2008).

In a stroke model of disease, an aqueous extract of Indian ginseng roots significantly exhibited the better short-term functional outcomes in locomotor activity (Raghavan & Shah, 2015). These beneficial effects have been considered to be due to active constituents named as withanolides which are steroidal lactones in nature. This plant also possesses withaferin A and sitoindosides VII-X which have been reported to have effective anti-oxidant effets (Sözmen *et al.,* 2001). Taking into mind the potential effects of this plant in various neurological diseases, this study was planned to assess its role in accelerating functional recovery following nerve injury. For this, we utilized our previously established model of sciatic nerve lesion to find out its role in influencing the functional recovery.

Materials and methods

Animals

This project was carried out in mice (BALB/C). Animals were arranged from the Department of Physiology, Government College University Faisalabad. The average age of animals was 8-10 weeks and their body weight was 25-35g. All mice were kept in polycarbonated cages as 1 mouse/cage and acclimatized to the environment, at a controlled temperature of 25±2°C, ambient humidity (40-60%)

and ad-libitum supply of food and drinking water. 12-hour day and dark light cycle was upheld. All experiments were done during light cycle.

Collection and Processing of Plant

Indian ginseng roots were procured from local market and identified by taxonomist from the Department of Botany, Government College University, Faisalabad. Roots were ground into fine powder. For extraction, the half quantity of powder was soaked into n-Hexane while second half was soaked into Chloroform for 10 days. Later on, both extracts were filtered using filter paper and filtrates were then evaporated using rotary evaporator.

Nerve Compression

Sciatic nerve compression was done by following the protocol as previously mentioned (Hussain *et al.,* 2013, Zafar *et al.,* 2020). Briefly, following a week of acclimatization, every mouse was sedated with the blend of xylazine (5mg perkg body weight) and ketamine (70mg perkg body weight) which was injected intraperitoneally. For the purpose of inducing nerve injury, the SN (sciatic nerve) of right leg at mid-thigh region was uncovered. The uncovered SN was precisely squashed by holding with forceps for 12-15 seconds and then skin was sutured using 4-0 acrylic suture. For a couple of days, pyodine was applied to the sutured region to avoid infection. Hind limb contra lateral to the lesion was served as intra-control (Halter *et al.,* 2010).

Study design

Following nerve compression, animals were equally divided into three groups i.e. control and two treatment groups. Control (n=4) group was offered plain diet without any addition of treatment in their diet while treatment group n.Hexane (n=4) was offered diet mixed with ginseng root n. Hexane extract at the daily dose rate of 100mg/kg body weight and treatment group Chloroform (n=4) was offered diet mixed with ginseng root chloroform extract at the at the daily dose rate of 100mg/kg body weight. Treatment groups were offered treatment containing diet since the day of nerve crush in animals.

Behavioral Analyses

Throughout the study period, following behavioral analysis were done before and after nerve injury induction at different time points in mice for the purpose of behavioral comparison among groups;

Body weight and Diet intake

Throughout the study period, daily body weight and diet intake consumption were measured for all experimental animals. Daily diet intake consumption was recorded by taking the difference between weight of offered diet and that of remaining diet in the cage on each day.

Walking Tract Analysis

This test was performed to assess the extent of recovery in motor functions following sciatic nerve crush lesion by adopting the protocol as mentioned in previous studies (Zafar *et al.*, 2020, Razzaq *et al.*, 2020). Briefly, the hind paws of the mouse were tinted with the nontoxic ink and then were allowed to walk on a wooden path of (7cm × 50cm) having (white paper floor) into a dark box. Foot prints of hind paws were obtained which were measured for the calculation of Sciatic functional index (SFI) using the following formula;

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

In the given formula PL is print length (from heel to top of third toe), TS is Toe spread (distance from 1st and the 5th toe) and the IT is the intermediate toe spread (distance between 2nd and 4th toe). E stands for experimental side i.e., ipsilateral hindpaw while N stands for normal i.e. contralateral hindpaw (Kamran *et al.,* 2020).

Grip Strength Test

Another authentic way to assess motor functions reclamation is grip strength analysis, a non-invasive technique which allows us to estimate the strength of hindlimb's muscles. This was done by following the protocol given in earlier studies (Zafar *et al.*, 2020) (Hussain *et al.*, 2013) by means of the grip-strength meter (Bioseb, Chaville, France). The test was measured for both hind limbs contralateral and

ipsilateral to the lesion which shows the gripping force in units of 'N' on screen. The mean of 3 readings were recorded for each mouse (Luca, 2008).

Hot Plate Test

Retrieval of sensory functions was assessed by observing the response of animal to thermal stimulus which was provided by positioning the animal's experimental paw on hot surface. This analysis was done using the previously described protocol (Zafar *et al.,* 2020). Concisely, each mouse was adapted to a non-functioning hot plate for a minute. Then, the temperature of plate was adjusted to 56 ± 0.5°C. Put the animal on hot surface in such a way that it's experimental paw was in direct touch with the hot surface. Monitored the animal for a while until it withdrew its paw, jerk or lick. Observed this time as paw withdrawal response for further comparison (Haas *et al.,* 2010b). Total of three readings were taken for the experimental limb with 2 minutes' interval. In case, there was no paw withdrawal response until 30 secs, removed the animal from hotplate and the paw withdrawal response was considered as 30 sec.

Biochemical Analyses

At the end of study period, all animals were euthanized, their blood was collected, centrifuged and serum was separated and stored for biochemical analyses which are as follows;

Total Oxidant Status

Total oxidant status (TOS) was measured in serum of mice to get an idea about the extent of oxidative stress in body following sciatic nerve lesion among all groups. The total oxidants existing in the serum sample were measured by adopting method given by Erel, 2005 using spectrophotometer (Biolab-310). The assay was calibrated with H_2O_2 and the results were expressed in terms of µmol of H_2O_2 equivalent/L (Erel, 2005; Maqbool *et al.*, 2020; Zafar *et al.*, 2020).

Total antioxidant capacity

Total antioxidant capacity (TAC) in serum samples, was measured by adopting the spectrophotometric method given by Erel, 2004 with few modifications to evaluate the oxidative stress combating efficacy in animals. This assay was calibrated by Vitamin C and the assay results were observed as mmol of Vitamin C equivalent/L (Erel, 2004) (Kamran *et al.*, 2020).

Muscle Mass Analysis

This analysis was done to measure and compare the muscle mass from ipsilateral and contralateral to the injury site in each animal. This test would predict about the extent of muscular dystrophy. Following euthenization, animals were dissected to harvest their muscles i.e. Tibialis anterior and Gastrocnemius, from both hind limbs of each animal. The level of muscle atrophy was checked and compared among all groups by measuring the muscle mass ratio (Gargiulo *et al.,* 2014).

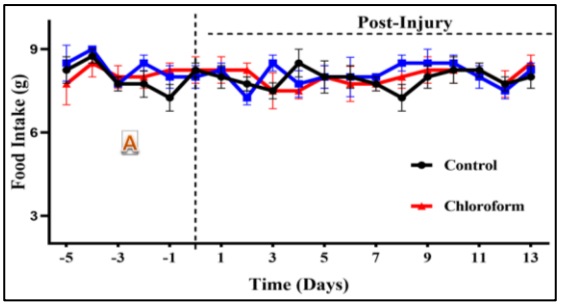
Statistical Analysis

All data were taken as mean ± standard error of mean (SEM) which were analyzed statistically using ANOVA followed by multiple comparisons test on GraphPad Prism, version 8.0 software. A value of $p < 0.5$ was considered significant to take final decision for each parameter.

Results

Impact of Indian ginseng extracts on diet intake and body weight

The daily diet consumption and body weight were recorded all over the study period. The variation in average body weight among all groups on each day was found to be non-significant (Fig. 1a). Similar pattern was observed for diet intake analysis i.e., non-prominent difference among all groups statistically (Fig. 1b). These findings indicate that the addition of both ginseng extracts in the diet did not alter the eating behavior of mice and mice grew in the way as they would under ordinary conditions.



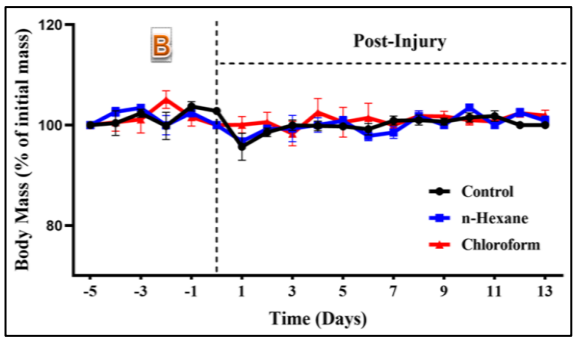


Fig. 1. Effect of ginseng extracts on diet intake and body mass; Results are presented as mean ± SEM (n=4)**.** (a) Presents time course of diet intake in mice served with routine diet (Control= black line), ginseng n.Hexane extract containing chow at the dose rate of 100mg/kg body weight (blue line) or Chloroform extract containing diet at the dose rate of 100mg/kg (red line). Two-way repeated measure ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction indicated non-significant differences among all groups at all-time points (p=0.844). (b) Presents time course of body weight of mice as in (a). Two-way repeated measure ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction indicated non-significant differences among all groups at all-time points (p=0.543).

Impact of Indian ginseng extracts on motor functional recovery

The motor functions regain following sciatic nerve injury was evaluated by muscle grip strength which was measured on different time points. We observed that both ginsengs extract treated groups showed earlier motor function regaining (Fig. 2a). The trend of graph presenting grip strength showed prominently higher grip force restoration on day 8, 10 and 12 post-injuries for both treatment groups. However, Chloroform group showed more significant enhancement in gripping force on day 10 and 12 post-injury among all. Similarly, Fig. 2b depicts prominent restoration in value of SFI, another indicator depicting motor function restoration, at day 9 and day 12 post-injury for both treatment groups. However, chloroform group revealed relatively improved SFI on day 9 and 12 as compared to n.Hexane group.

The sensory functions regain was observed in both treatment groups as assessed by hotplate test on different days. Immediately following the nerve injury, the sensitivity capability was decreased in all groups (Fig. 2c). A significant decrease in paw withdrawal latency was observed on day 7 post-injury (P=0.03) in both treatment groups which pointed towards the quicker sensory function reclamation in them as compared to untreated group (Fig. 2c).

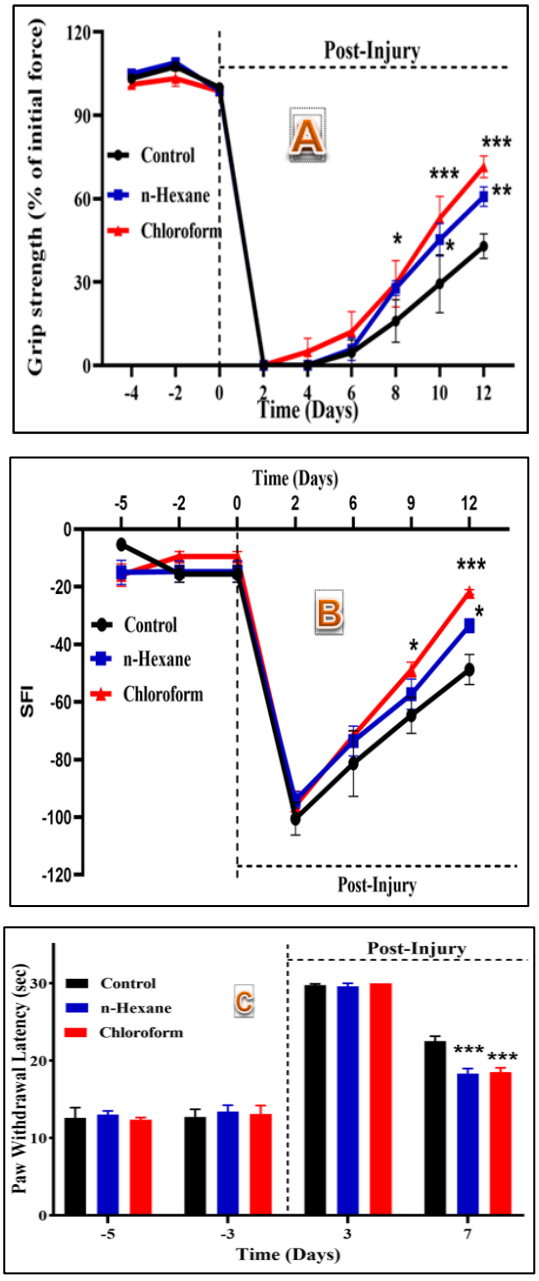


Fig. 2. Effect of ginseng extracts on sensorimotor functions revival; Results are presented as mean ± SEM (n=4)**.** (a) Presents time course of grip strength in mice served with routine diet (Control= black line), ginseng n.Hexane extract containing diet at the dose

rate of 100mg/kg body weight (blue line) or Chloroform extract containing diet at the dose rate of 100mg/kg (red line). Grip strength is expressed as percentage of mean of initial force taken at day -4 and -2 before nerve lesion. Two-way repeated measure ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction indicated highly significant differences for Chloroform group vs. Control (*p=0.01) on day 6 following injury, Chloroform vs. Control (***p<0.001), n.Hexane vs. Control (*p=0.01) on day 9 following injury and Chloroform vs. Control (***p<0.001), n.Hexane vs. Control (**p=0.001) on day 12 following injury. (b) Presents time course of Sciatic functional index (SFI) in mice as in (a). Two-way repeated measure ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction presented significant differences for Chloroform group vs. Control (*p=0.01) on day 9 following injury, Chloroform vs. Control (***p<0.001), n.Hexane vs. Control (*p=0.01) on day 12 after injury. (c) Presents time course of sensory response revival as measured by earlier paw withdrawal from hot surface in mice as in (a). Two-way repeated measure ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction presents highly significant differences for both treatment groups vs. Control (***p<0.001) on day 7 following nerve lesion.

Impact of Indian ginseng extracts on oxidative stress

The oxidative stress, pathological response to any kind of injury, was measured in all groups which was included the evaluation of TOS and TAC levels in serum samples. A significant increase in TAC values (*P=0.01) was observed in both treatment groups (fig. 3b) which indicates towards the possible potential of both extracts in controlling the oxidative stress by increasing the antioxidant capability in body. Similarly, a significant decrease in TOS (*P=0.03) has been observed in Chloroform extract treated group (Fig. 3b). n.Hexane treated group also showed graphically reduced TOS value but statistically it is non-significant. Overall, these findings indicate the posible role of this plant in combating the oxidative stress.

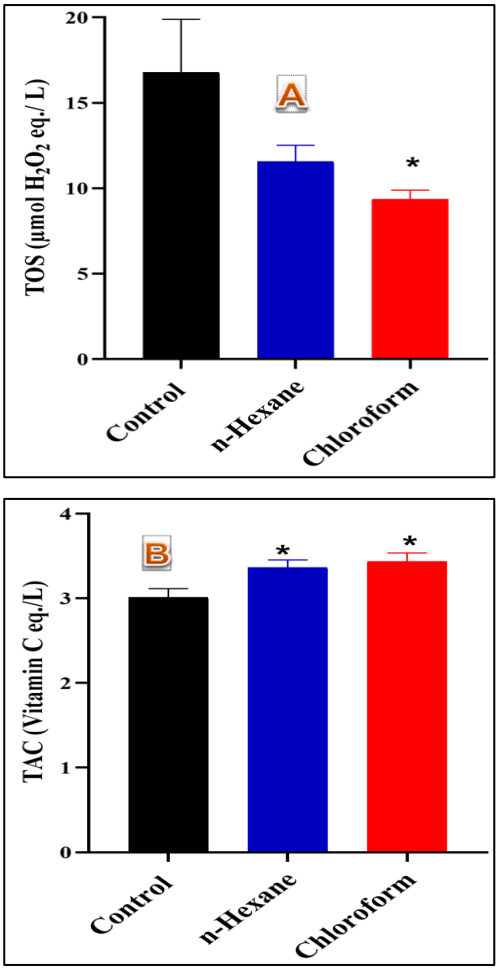


Fig. 3. (a) Total oxidant status and (b) Total antioxidant capacity measured in animals (n=4) fed on rutine diet (black bar) or different extracts of Indian ginseng containing diet i.e. 100mg of each extract are indicated by blue (n-Hexane) and red (Chloroform) bars. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction revealed a significant difference between Chloroform vs. Control (*p=0.02) and non-significant difference between n.Hexane and Control (p>0.05) for TOS while significant differences between Control and both treatment groups (*p=0.1) for TAC.

Impact of Indian ginseng on muscle mass

Because, the peripheral nerve abrasion/injury leads to the muscular denervation which eventually cause condensed muscle mass. The restoration in muscle mass of affected limb indicates the recovery of injured nerve functions through reviving its conduction of impulses to the muscles.

Hence the muscle measurement is another parameter to support the evidence of functional recovery. The muscle mass ratios of gastrocnemius and anterior tibialis (calculated by dividing muscle mass of ipsilateral to that of contralateral) muscles of all groups were compared and presented in Fig. 4a and b. Comparatively improved mass ratios for both muscles in both treatment groups indicate that treatments have played role in accelerating the functions revival. Chloroform extracts group showed statistically prominent increase in muscle mass which pointed towards comparatively more potential of this extract in enhancing the functional revival.

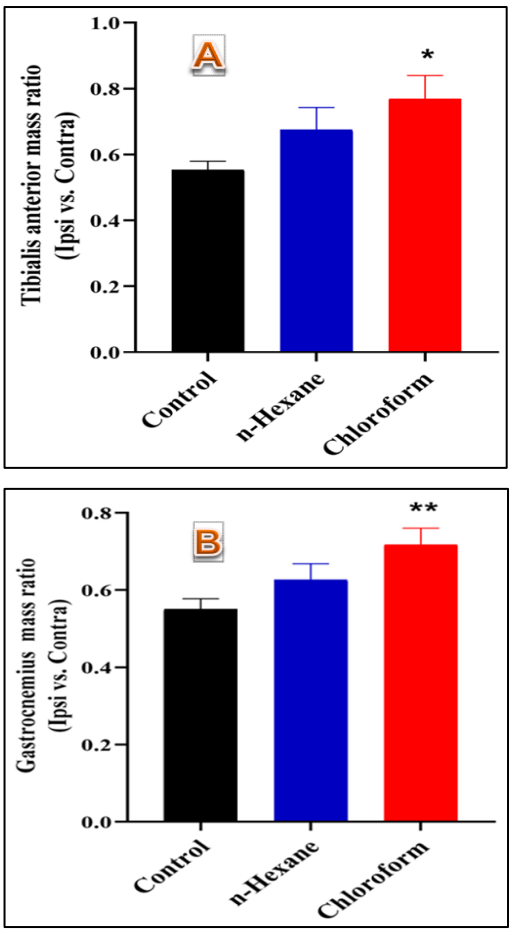


Fig. 4. (a) Tibialis anterior and (b) Gastrocnemius mass in animals (n=4) fed on routine diet (black bar) or different extracts of Indian ginseng containing diet i.e. 100mg/kg/day of each extract as indicated by blue (n-Hexane) and red (Chloroform) bars. Measurements have been expressed as a ratio between hind limbs ipsilateral and contralateral to the lesion. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test along with Benjamini, Krieger and Yekutieli's correction revealed a significant difference between Chloroform vs. Control (*p=0.02) and non-significant difference between n.Hexane and Control (p>0.05) for Tibialis mass ratio while significant differences between Control and Chloroform (**p=0.001) and non-significant difference between control and n.Hexane (p>0.05) for Gastrocnemius mass ratio.

Discussion

Indian ginseng belongs to the Solanaceae/nightshade family and has many common names like poison gooseberry, ashwagandha and Indian ginseng. It is a commonly used plant which has been used in medicine since ancient times because of its polypharmacological nature (Singh *et al.*, 2015). Moreover, it is used as anti-cancer, anti-inflammatory, nerve-tonic, and aphrodisiac (Kuboyama *et al.*, 2005). Importantly, the presence of its active constituent named as withanolides, has been considered to be associated with its many biological activities. In addition, effective role of this plant in numerous models of neurological disorders including PD, AD, HD, stress and epilepsy (Kulkarni & Dhir, 2008). Similarly, in a stroke model of disease, an aqueous extract of Indian ginseng roots at the dose of (200mg/kg) significantly exhibited better short-term functional outcomes in locomotor activity. Thus its ability to reverse the functional outcomes followed by the stroke onset is an important indicator of functional recovery (Raghavan & Shah, 2015). Based on these pieces of evidence, it can be stated that Indian ginseng is pivotal in terms of neuroprotection along with its ability to act as an anti-oxidative and anti-inflammatory agent. The present study was decided to evaluate its therapeutic role in a mouse model of peripheral nerve injury.

Normally, addition of any drug in diet of animals can change the taste or smell which ultimately alter the animal's preference for taking that diet. In current study, the plant extracts were administered through mixing in animal's diet. Statistically non-significant differences among groups for both body weight and diet intake analyses implicate normal

food intake behavior of all animals which points out that food consumption was not affected by addition of Indian ginseng.

Indian ginseng has been observed to improve the muscle grip strength revival in many CNS pathological conditions (Durg *et al.*, 2015). In current scenario, grip strength was also found to be improved due to Indian ginseng. Data was comparable in the grip strength of control group and ginseng extracts treated groups from day 6, day 9 and 12 post-injury. These results are relatable with the study of (Wankhede *et al.*, 2015), which support the effect of Indian ginseng supplementation on muscle strength, muscle size and recovery. The significantly improved data of SFI on day 9 and 12 in treatment groups showed accelerated functional recovery which further supports the potential of ginseng extracts for speedy motor function revival.

The nerves that control the muscles can be damaged by an injury, which can result in muscle atrophy. Extract from Indian ginseng contains compounds that promote neurite growth in mice (Kuboyama *et al.*, 2005; Kuboyama *et al.*, 2006). In another anabolic study, the mice treated with Ashwagandha showed anabolic activity (Grandhi, Mujumdar, & Patwardhan, 1994). These studies suggest the positive impact of ginseng on muscles mass. In this project, the significantly improved muscle mass ratio of the Tibialis anterior and Gastrocnemius in treatment group with ginseng chloroform extract suggest and support the idea that this plant has capability to prevent muscular atrophy.

Indian ginseng fruit extract was examined as a therapeutic drug which show the potential to tunes sensory responses and coordination of psychomotor function (Pingali, Pilli, & Fatima, 2014). Findings of this study have been appeared to show significantly improved sensory recovery of the lost sensory responses in case of injured sciatic nerve which further suggest and backing the potent role of ginseng root extract in enhancing the sensory function reclamation. The significantly improved values of TAC along with reduced TOS in ginseng chloroform treated group suggest that the observed beneficial effects of Indian ginseng can be due to its anti-oxidative ability (Durg *et al.*, 2015). The root of the plant Indian ginseng is known to increases antioxidant properties as evidenced by previous studies (Alam *et al.*, 2012). Earlier studies have reported that lipid peroxidation was inhibited in stress-induced animals with the use of Indian ginseng (Dhuley, 1998). With regard to the results of this study, it can be assumed that Indian ginseng could be the possible source for speeding up injured nerve regeneration but it needs to be elucidated further.

Conclusion

Thus, in current circumstance, it can be ventured that Indian ginseng root's Chloroform extract has the impending role in escalating sensorimotor functions recapture following sciatic nerve lesion. It can be proposed that this effect may be due to oxidative stress fighting role of the ginseng extract. Though, further studies should be conducted to seek out all possible mechanistic ways behind this upgraded functional retrieval. It is agreed that this extract may have numerous chemical constituents. Therefore, it is suggested to explore and characterize these compounds which could be potently playing role behind this accelerated functional recovery following PNI.

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