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Rhoifolin Provides Neuroprotection against Spinal Cord Injury by Attenuation of Oxidative Stress, Inflammation and Apoptosis in Rats

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ABSTRACT

Background: Rhoifolin is a plant flavonoid known to have antioxidant and anti-inflammatory properties. This study was taken to identify the effect of rhoifolin on Spinal cord injury (SCI) in a rat model.

Methods: Motor function of SCI rats was carried out by using in Basso, Beattie and Bresnahan test. Expression levels of different proteins such as IL-1β, IL-6, TNF-α, NF-κB p65, p-IκB-α, p38MAPK, caspase-3 and caspase-9 were carried out by using western blotting analysis. The Concentration of proteins was determined by using Bradford's method. Estimating of Glutathione (GSH), Glutathione peroxidase (GPx) and Superoxide dismutase (SOD) was carried out by using commercially available kit.

Result: Rhoifolin was tested at 20 and 40 mg/kg doses and showed a significant improvement in the motor function of SCI rats in Basso, Beattie and Bresnahan test. Moreover, rhoifolin treatment also attenuated pro-inflammatory cytokine levels such as IL-1β, IL-6 and TNF-α. The NF-κB pathway showed a significant attenuation as evident in the significant downregulation of the NF-κB p65 and p-IκB-α levels. In addition, rhoifolin treatment also caused a significant reduction in oxidative stress, as evident from changes in intracellular levels of Glutathione, GPx, MDA and SOD in the articular cartilage tissue. Further, we also tested rhoifolin for its effect on the apoptotic pathway by estimating the intracellular levels of p38MAPK, caspase-3 and caspase-9. The current results show that rhoifolin has a neuroprotective in SCI rat model.

Key words: Cytokines, NF-κB pathway, Oxidative stress, Rhoifolin, Spinal cord injury.

INTRODUCTION

Spinal Cord Injury (SCI) is often caused by a physical injury that leads to infiltration of inflammatory cells and secondary degeneration (Ahn *et al.*, 2015; Shin *et al.*, 2013). SCI results in devastating impairment of neurological functions such as autonomic, motor and sensory dysfunction. Secondary injury in SCI results in lesions spreading out of the primary injury site leading to further damage (Aslan *et al.*, 2009). The secondary injury in SCI is marked by increased inflammation and oxidative stress. Increased oxidative stress occurs when the level of reactive oxygen species (ROS) is more than the capacity of the antioxidant mechanism to scavenge them (Khayrullina *et al.*, 2015; Paterniti *et al.*, 2009). Moreover, the heightened production of inflammatory agents causes a strong inflammatory response (Genovese *et al.*, 2009; Ni *et al.*, 2014). It has been shown that attenuation of inflammatory signal helps in SCI recovery (Chen and Jin, 2016; Ji *et al.*, 2014; Machova Urdzikova *et al.*, 2016).

Mitogen-activated protein kinase (MAPK) is a factor in the signal transduction cascade for apoptosis (Pereira *et al.*, 2013). The MAPK levels are generally a determiner of cell survival or death since their level reflects cell damage (Nafees *et al.*, 2015; Yamaoka *et al.*, 2012). Upregulated expression of MAPK has been reported in the glial cells and neurons in SCI (Ha *et al.*, 2011; Lee *et al.*, 2010). Moreover, MAPK is also involved in the inflammatory response (Bretón-Romero and Lamas, 2013).

The extant treatment regimens for SCI are largely ineffective and fail to result in complete recovery from neurological and functional impairments (Varma *et al.*, 2013). Flavonoids are bioactive compounds of plant origin with a 15-carbon skeleton including two fused 6-carbon rings (Merken and Beecher, 2000). Flavonoids in the human diet constitute a common group of polyphenolic compounds produced in plant cells as secondary metabolites. Flavonoids contain phenolic hydroxyl groups that mediate their antioxidant activity (Rice-Evans *et al.*, 1996; Zheng *et al.*, 2009), anti-inflammatory (Nijveldt *et al.*, 2001) and the ability to scavenge free radicals (Cho *et al.*, 2013). Flavonoids are

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known to show therapeutic potential in SCI (Zhang *et al.*, 2017).

Rhoifolin is a flavanone first extracted from *Rhus succedanea* (Hattori and Matsuda, 1952). Rhoifolin has been shown to have anti-inflammatory, antioxidant (Eldahshan and Azab, 2012) and anticancer (Eldahshan, 2013) activity. Oxidative stress has an important role in progressing neurodegenerative disorders (Valko *et al.*, 2007). Therefore, the antioxidative properties of rhoifolin may mediate its antioxidant and anti-inflammatory in SCI. Hence, the current study was planned to investigate the neuroprotective effect of rhoifolin on a rat model of SCI.

MATERIALS AND METHODS

Experimental animals and estimation of rhoifolin toxicity

Male Sprague-Dawley rats (2 months old, weighing around 190-210 g) were procured from the institutional animal housing facility. The experimental animals were kept under 12/12 h light/dark cycle under controlled conditions. The rats were fed with the standard rat diet and R.O. water as required. The animal experiments were approved by the Institutional Review Board of Shanghai University of Traditional Chinese Medicine and Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Approval No. JMU022/2019-22). This work was carried out in Shanghai University of Traditional Chinese Medicine from 2019-2021.

Rhoifolin (Sigma Aldrich USA) was dissolved in PBS and was administered in graded doses of 20, 40, 60, 80 and 100 mg/kg via the intraperitoneal route to five rats each as mentioned by Zhao and his colleagues (Zhao *et al*., 2021). The animals were kept on a 10-day observation period. Health parameters such as diet, changes in weight, fluid intake and psyco-motor changes were measured. Rhoifolin did not show any toxicity at all the tested doses. Therefore, we selected the lowest two doses *i.e.* 20, 40 mg/kg for further experiments.

Establishment of rat SCI model and experimental groups

For surgery, the rats were anesthetized by intraperitoneal administration of ketamine (40 mg/kg) and xylazine (5.5 mg/kg). Laminectomy of T_8 and T_9 was performed to remove vertebral peduncles and moderate contusion injury was executed using a modified Allen's weight drop apparatus on the exposed spinal cord with a weight of 10 g at a height of 55 mm. Mechanical compression was confirmed by retraction flutter of legs, tail spasm and delayed paralysis.

The animals were randomly assigned to six experimental groups of 10 rats each: (1) Healthy group, no surgery (2) control group, with laminectomy + PBS (i.p.) without SCI (3) SCI group, SCI+PBS(i.p.) (4) MP group, treated with 55 mg/kg methyl prednisolone (i.p.) once a day for 5 days (5) 20 mg/kg rhoifolin, treated with 20 mg/kg rhoifolin (i.p.) once a day for 5 days (6) 40 mg/kg rhoifolin group, treated with 40 mg/kg rhoifolin (i.p.) once a day for 5 days. The experimental groups were established 12 hours after the establishment of SCI.

Locomotor function test

Locomotor activity was estimated by Basso, Beattie and Bresnahan (BBB) score method (Basso *et al.*, 1995). BBB is a well-established method for the study of motor function after spinal cord contusion where a lower BBB score indicates a greater impairment of motor functions. The BBB scores range from 0, signifying no hind limb movement to 21 signifying normal motor function. The BBB scores of the experimental groups were estimated at 24, 48, 72 hours and 7 days after spinal cord contusion. The technicians conducting BBB test were blinded to the group assignment using random codes.

Estimation of water-content in the spinal cord

The water-content in spinal cords was estimated after the commencement of drug treatment. The animals were euthanized with a 500 mg/dose of ketamine (IM) and spinal cords were excised and weighed. For dry weight, the spinal cord samples were dried for 24 h at 80°C. The percentage of water-content in the spinal cords was calculated using the formula:

$$\frac{\text{Wet weight - Dry weight}}{\text{Wet weight}} \times 100$$

Estimation of cytokine levels

Blood was drawn by retro-orbital puncture of all experimental groups at the end of drug treatment scheduled. The blood was stored in anticoagulant and centrifuged at 3000g for 30 min to collect the serum. The levels of TNF-α, IL-1β and IL-6 in the sera of CFA induced animals using ELISA kit (sigma bioscience, USA), following the manufacturer's instructions.

Estimation of oxidative stress

The animals were euthanized with a 500 mg/dose of ketamine (IM) and spinal cords were excised. 20 mg of spinal cord tissue were taken and homogenized in PBS and centrifuged at 12500 g for 30 min. The protein content of tissue supernatants was estimated using the Bradford's method and equal amount of protein was used for estimating the concentration of, Glutathione (GSH) using Glutathione GSH/GSSG Assay Kit (Sigma Aldrich, USA), Glutathione peroxidase (GPx) using Glutathione assay kit (Cayman Chemicals, USA), Malondialdehyde (MDA) by MDA assay kit (Abcam, USA) and Superoxide dismutase (SOD) by Superoxide Anion Assay Kit (Sigma Aldrich, USA). All the estimations were carried out following the respective manufacturer's protocols.

Western blot analysis of MAPK

Antibodies for NF-κB-p65, p-NF-κB-p65 and p38MAPK were procured from Santa Cruz Biotechnology, Inc. (USA). The anti-IκB-α and anti-p-IκB-α antibodies were purchased from the Cell Signaling Technology, Inc. (USA). An equal amount of total protein from spinal cord supernatants (described above) was electrophoresed in SDS-PAGE gel in three replicates and electrophoretically transferred on PVDF

membranes. The membranes were washed with Tris-buffered saline which contained 0.2% Tween 20 (TBST) and blocked with 4% Non-fat dry-milk. Subsequently, primary antibodies were added with new blocking solution and incubated for 4 hours. HRP was added to the membranes after washing with Tris buffer saline. The membranes were developed with ECL detection system. The relative intensity of bands was analysed using ImageJ software (NIH, USA).

Estimation of Caspase-3 and caspase-9 levels

An equal amount of total protein from spinal cord supernatants was added with reaction buffer containing respectively Ac-LEHD-pNA and Ac-DEVD-pNA for caspase-9 and caspase-3. The tubes were incubated for 2 h at 37°C. Subsequently, the absorbance of reaction mixtures was recorded 405 nm on a spectrophotometer. The absorbance of treatment groups was compared with that of control groups.

Statistical evaluation

Individual mean±SEM were calculated from the data and the variance between the experimental groups was estimated using one-way ANOVA with Bonferroni's multiple corrections for post-hoc analysis. Significance values of $P<0.05$ were considered significant. All the statistical estimations were performed in Graph Pad Prism software.

RESULTS AND DISCUSSION

Effect of rhoifolin on locomotor function and spinal cord water-content

The locomotor function of experimental groups was determined using BBB scores (Fig 1). The BBB score was significantly reduced in the SCI groups comparing to the control in all the observation time points. A significant increase in BBB scores was observed in rhoifolin and MP groups in comparison to the SCI group. However, there was an insignificant difference in BBB scores between the rhoifolin groups as well as between the rhoifolin groups and the MP group.

The water content of spinal cord was estimated in the experimental groups (Fig 2). The SCI group had a significantly high water-content in comparison to the control group. Rhoifolin and MP groups and showed a significant decrease in spinal cord water content as compared to the SCI groups. However, there was o significant difference between the water content of rhoifolin groups and the MP group.

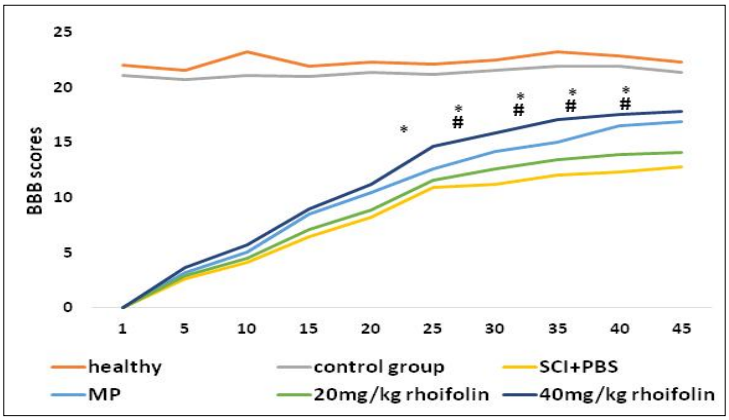

Fig 1: Effect of rhoifolin on BBB scores of SCI rats. *P<0.05 represents the comparison between the 20 mg/kg rhoifolin-treated group and the vehicle-treated group; #P<0.05 represents the comparison between the 40 mg/kg rhoifolin-treated group and the vehicle-treated group.

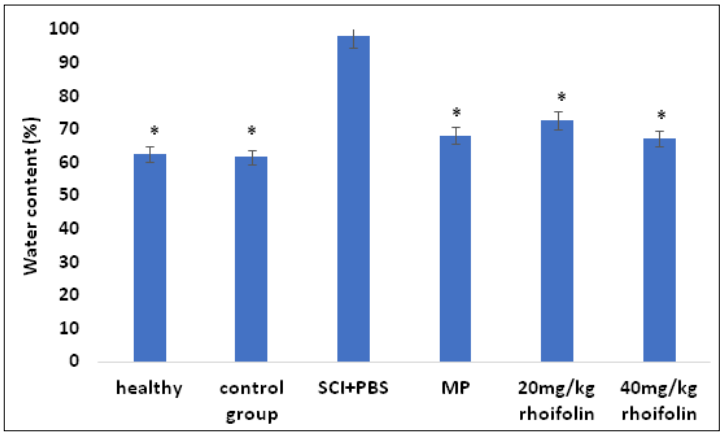

Fig 2: Effect of rhoifolin on spinal cord water content. *P<0.05 vs. SCI group.

Increased infiltration of proinflammatory cytokines and inflammatory response is a hallmark of the secondary injury in SCI. Rhoifolin treatment showed a significant attenuation of pro-inflammatory cytokine levels in SCI rats. The transcription factor NF-κB is a key mediator of inflammatory responses (Makarov, 2001). NF-κB also helps in the amplification of the inflammatory signal on the account of a cytokine feedback loop. Therefore, our results indicated that the anti-inflammatory and antioxidative properties of rhoifolin in SCI induced arthritis model are mediated by the NF-κB pathway. Previously rhoifolin has been reported to reduce inflammation in the rat edema model where it showed a significant attenuation of TNF-α levels (Eldahshan and Azab, 2012). Therefore, our findings anti-inflammatory effect of rhoifolin is consistent with previous studies.

Effect of rhoifolin on pro-inflammatory cytokine and antioxidants levels

ELISA analysis showed that the induction of SCI caused significantly increased blood sera TNF-α, IL-1β and IL-1 levels in comparison to the control (Fig 3). However, rhoifolin treatment caused a significant reduction of these cytokines as compared to the SCI group. It was also observed that cytokine levels in the highest rhoifolin concentration group were similar to the MP treatment group.

SCI induction significantly increased the oxidation levels in the spinal cord tissue (Fig 4). The levels of GSH, GPx, SOD was significantly downregulated and the levels of MDA was upregulated in SCI animals in comparison to the control. Rhoifolin treatment showed a significant improvement in the oxidation state in the articular tissue as compared to the SCI group animals. Moreover, the highest rhoifolin concentration group (40 mg/kg rhoifolin) showed an oxidation state at par with the MP group.

In addition to pro-inflammatory cytokines, oxidative stress is also a major factor in the progression of secondary injury in SCI (Khayrullina *et al*., 2015; Paterniti *et al*., 2009). The existence of high levels of ROS also indicates the degree of injury to the spinal cord and alleviation of oxidative stress has been suggested for therapeutic intervention in SCI (Jia *et al*., 2012). In this study, we used Glutathione, GPx, MDA and SOD levels as markers for the estimation of oxidative stress in the articular chondrocytes. Rhoifolin showed a strong antioxidant response in SCI animals, as evident from a significant decrease in these oxidative stress levels in the rhoifolin treatment groups. Previously, several other flavonoids have been reported to show antioxidative properties in SCI (Juurlink and Paterson, 1998). Therefore, we propose that rhoifolin can be as effective as other previously proposed flavonoids for SCI treatment.

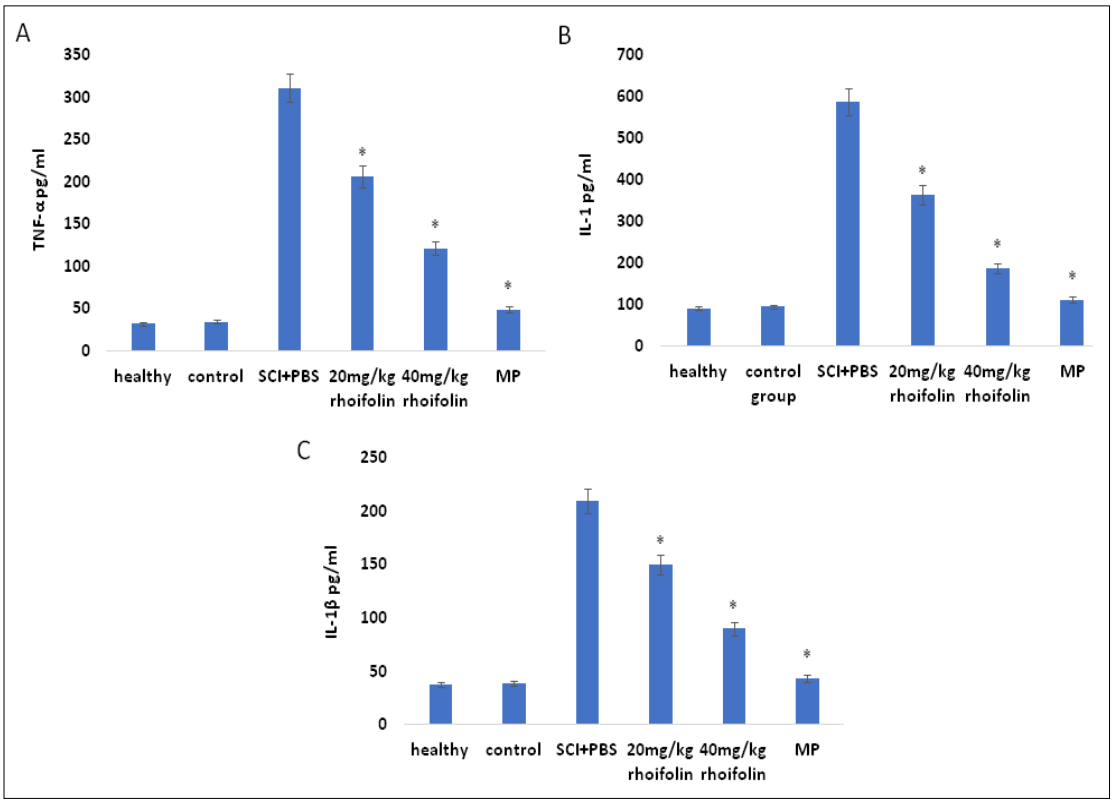

Fig 3: Effect of rhoifolin treatment levels of TNF-α (A) IL-1(B) IL-1β (C). Data are expressed in mean±SEM. *P≤0.05 compared to SCI induced group.

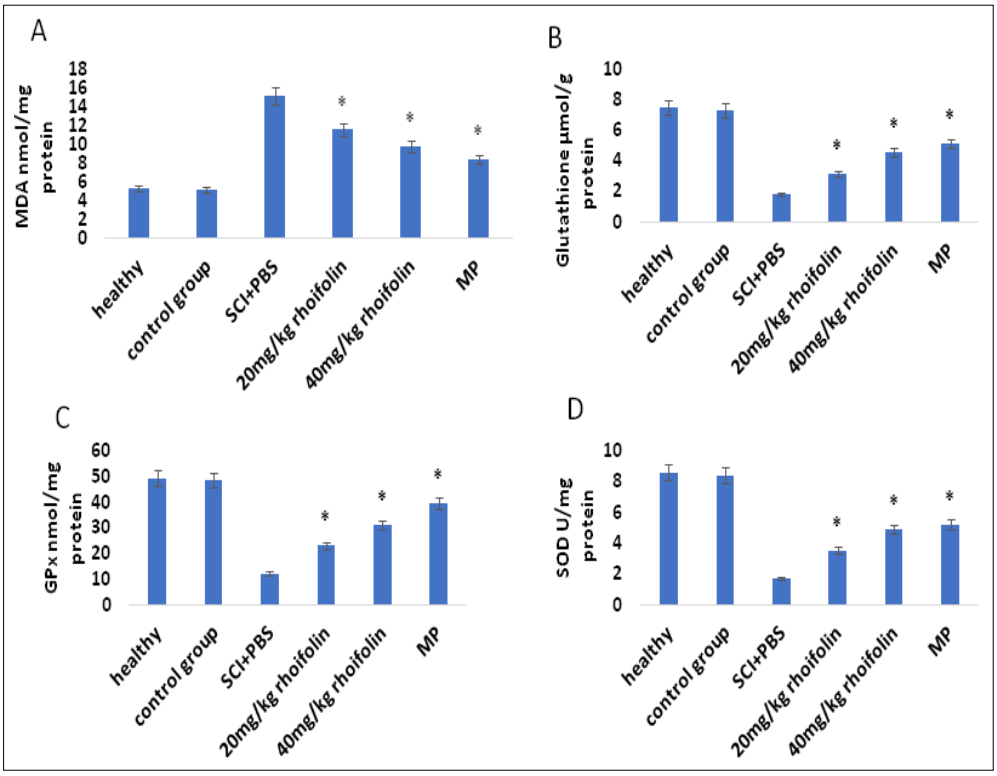

Fig 4: Effect of rhoifolin treatment levels of MDA (A) Glutathione (B) GPx (C) SOD (C) SOD (D). Data are expressed in mean±SEM. * P≤0.05 compared to SCI group.

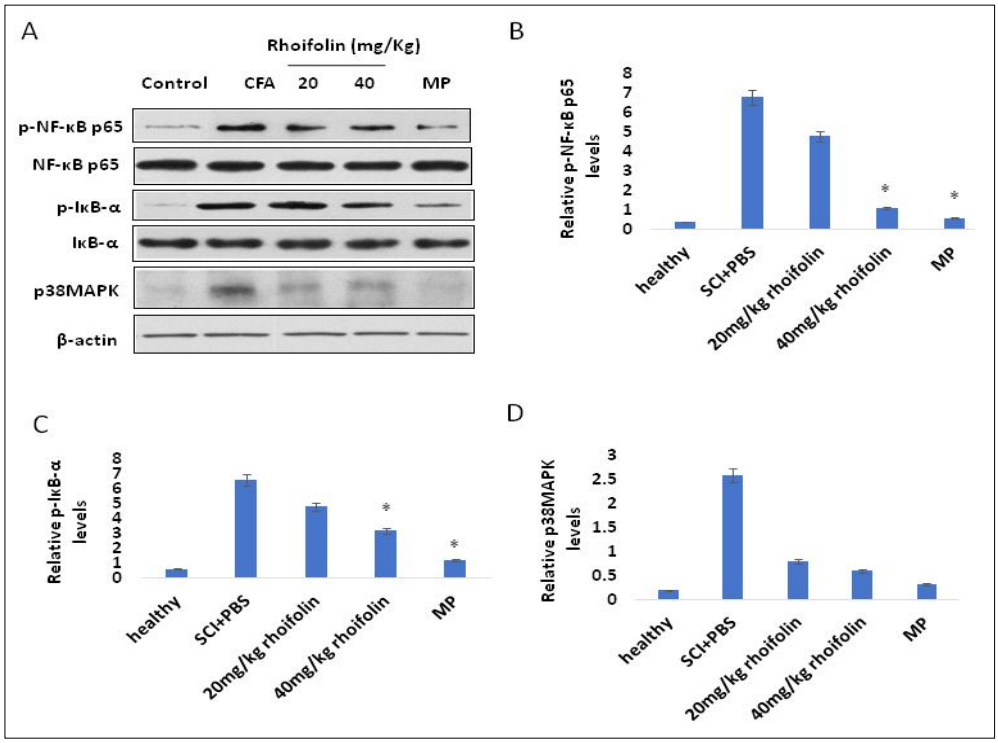

Fig 5: Western blot analysis of levels of phosphorylated and non-phosphorylated forms of NF-κB p65 and IêB-á and p38MAPK with β-actin used as a control (A). Western blot densitometry analysis of phosphorylated NF-êB p65 (B) Western blot densitometry analysis of phosphorylated IêB-á (C) Western blot densitometry analysis of p38MAPK (D). Data are expressed in mean±SEM. * P≤0.05 compared to CFA induced group.

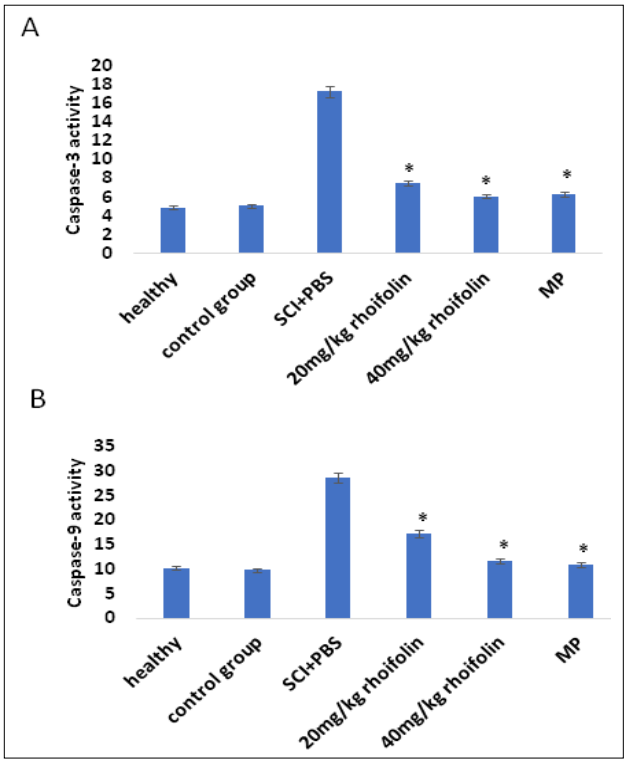

Fig 6: Effect of rhoifolin on Caspase-3 (A) and Caspase-9 (B) levels. Data are expressed in mean±SEM. *P£0.05 compared to CFA induced group.

Rhoifolin attenuates MAPK levels and downregulates caspase activity in SCI rats

The effect of rhoifolin on the transcription factor NF-κB and p38MAPK was tested on the experimental groups. The western blot analysis showed a significantly large increase NF-κB-p65 and IκB-α and p38MAPK levels in spinal cord (Fig 5). Treatment with rhoifolin significantly decreased the levels of phosphorylated forms of NF-κB, IκB-α and p38MAPK.

Caspase-9 and caspase-3 activity was estimated in the experimental groups (Fig 6). These activities showed a significant upregulation in the SCI group in comparison to the control group. Rhoifolin treatment showed significant downregulation in the caspase activity. Moreover, the caspase activity in rhoifolin group was at par with that of MP group.

P38MPK is present in an inactive state in the cytoplasm of cells and is transported to the nucleus when activated (Tang *et al.*, 2013). The phosphorylation cascade for activation of p38MAPK is triggered by external stimuli such as pro-inflammatory cytokines and free radical injury (Brunetti *et al.*, 2013; Park *et al.*, 2015; Zhu *et al.*, 2013). The activated p38MAPK upregulates the expression of iNOS, COX and other inflammatory molecules resulting into the amplification of the inflammatory signal (Bretón-Romero and Lamas, 2013). Flavonoids have previously been suggested for arresting MAPK signaling in neurodegeneration (Schroeter *et al.*, 2002). In this study, treatment with rhoifolin resulted in a significant downregulation of p38MAPK indicating attenuation of the pro-apoptotic pathway. Moreover, this study also showed a significant reduction in caspase levels in SCI rats. SCI results in the induction of caspases which causes induction of apoptotic signaling pathway (Springer *et al.*, 1999). Previously, the flavonoid apigenin has been reported to attenuate the levels of caspase-3 in SCI (Zhang *et al.*, 2014). Therefore, we propose that rhoifolin is can be as effective as other flavonoids in the treatment of SCI.

Conclusion

In the present study, rhoifolin inhibited pro-inflammatory cytokines and the NF-κB pathways. Moreover, the intracellular oxidative stress was also significantly reduced upon rhoifolin treatment in SCI rats. This may have caused a reduction in apoptotic signal as observed in a reduction in the levels of p38MAPK and caspases. Overall, there was an improvement in the motor function in rhoifolin treated SCI rats. Therefore, the results of this study clearly show that rhoifolin can be a potentially safe alternative to extant therapies for SCI treatment. However, future studies will be mandated to identify the underlying mechanism of rhoifolin action on SCI such as the identification of its target molecules.

Conflict of interest: None.

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