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Evaluation of the neuroprotective effect of alcoholic extract of Achillea santolina L. flower on the degeneration of spinal cord alpha motor neurons after sciatica nerve injury in rat

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Abstract

As a consequence of peripheral nerve damage, the material released from the lesion received by the cell body of neurons in the central nervous system leads to apoptosis. As Achillea santolina L. has anti-inflammatory effects, it may reduce the severity of the lesion. Therefore, in this study, we investigated whether the alcoholic extract of Achillea santolina had neuroprotective effects on the degeneration of spinal cord alpha motoneurons after sciatica nerve injury in Rats. Twenty-four male Wistar rats were randomly divided into four groups: compression, compression and treatment with 50 mg/kg, compression in addition to treatment with 75 mg/kg, and a group without any intervention as a control. In groups of compression and treatment, the sciatic nerve was compressed for sixty seconds by artery forceps. After compression, the extract of Achillea santolina was injected intraperitoneally during the first and second weeks. 28 days later, the rats were sampled from the lumbar spinal cord. Comparing neuronal density in each group was done with the compression group. In the compression group, the density of alpha-motoneurons showed a significant decrease compared to the control group, and in the treatment groups of 50 and 75 mg/kg, the density of alpha-motoneurons increased significantly compared to the compression group. These findings showed that Achillea santolina alcoholic extract of flowers has compounds that due to anti-inflammatory and antioxidant factors have restorative and neuroprotective effects on spinal cord alpha-motoneurons after lesion and a dose of 75 mg/kg has the greatest effect in preventing the severity of degeneration.

Keywords: Peripheral nerve, Achillea santolina L., Nerve regeneration

1. Introduction

Peripheral nerve disturbances are prevalent neurological disorders that can be caused by various factors such as traumatic damage, tumors, and/or iatrogenic lesions. Loss of Motor, sensorial, and autonomous function can be affected by peripheral nerve damage, resulting in significant financial losses and reduced quality of life [1]. Despite optimal surgical

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repair, sensation and motor function may still be impaired [2]. Mechanisms of repair after injury of the peripheral nervous system differ significantly from that of the central nervous system; however, the preliminary success of antioxidants in inversing damages to the central nervous system may provide clues as to the pharmacological modulation of the repair of the peripheral nervous system [1]. Before nerve regeneration occurs, many degenerative processes occur, many of which provide a basis for regeneration. The distal segment of the damaged nerve undergoes calcium-mediated Wallerian degeneration, and the initial histological changes, including the physical fragmentation of axons and myelin, begin in the early hours of injury, which is essential for subsequent regeneration processes. Schwann's cells play a key role in Wallerian repair. Schwann's cells are active spontaneously 24 hours after injury, the cytoplasm and nucleus appear, and the rate of mitosis increases [3]. These cells divide rapidly to form differentiated daughter cells that express the genes involved in repair and regeneration at a high level. Many primary inflammatory cytokines are secreted in the endoneurium within a few hours, most of which are produced by Schwann's cells, and begin to increase in the distal nerve segment. A number of these proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-a) and interleukin-1b (IL-1b), have been implicated in the process of Wallerian degeneration, which is up-regulated primary and transiently at the location of nerve injury and they trigger local inflammatory responses. As a result of the excessive production of TNF-a and IL-1b, secondary damage occurred due to the release of other cytokines. Researchers found that instant treatment with an antagonist of TNF-a enhanced axonal regeneration after peripheral nerve injury [1, 4-9]. These findings demonstrated that drug combinations with anti-inflammatory effects could offer new therapeutic alternatives to limit the overabundance of inflammatory cytokines after peripheral nerve injury. Plants provide a wide range of secondary metabolites that have medicinal applications. Approximately 2/3 of the new chemicals identified each year are derived from higher plants. 25% of US pharmaceuticals are made from plants, which are based in the chemical synthesis industry [10]. One of these compounds is the extract of Achillea santolina. It is represented in the Northern Hemisphere by around 115 species from the genus Achillea (Family Asteraceae), most of which are found in Europe and Asia [11]. This plant is one of the most famous medicinal plants found in traditional medicine, which was widely used to treat diseases, especially rheumatic pain, inflammation, burns, wounds, etc. Among the secondary metabolites found in the genus are terpenes, polyphenols, flavonoids, and others. In a variety of cell and animal models, Achillea plants and their essential oils were found to have potent antibacterial, antiproliferative, and anticancer properties as an anti-inflammatory and for anxiety treatment, has been traditionally used in Iran this plant, also it is effective against conditions such as ulcerative colitis, primary dysmenorrhea, multiple sclerosis (MS), episiotomy wounds, irritable bowel syndrome (IBS), episiotomy wounds, oral mucositis, etc. [12-14]. Several studies have shown that the alcohol extract from Achillea santolina has anti-diuretic, and analgesic effects and that it reduces edema, hyperalgesia, and serum IL-6 levels, and makes morphine more effective as an analgesic [15-18]. Due to the presence of phenolic compounds and flavonoids, A.santolina hydroalcoholic extract has been shown to have antioxidant activity in fat peroxidation, superoxide oxidation, and catalase [19]. Alcohol dissolves Borneol, which is neuronal protective and anti-apoptotic [20]. The purpose of this research is to investigate the effects of the administration of Achillea santolina flower alcoholic extract in postponing or prevents the central degeneration of spinal motor neurons after rat sciatic nerve compression and thus improves the healing process.

2. Materials and Methods

2.1 Taking samples and preparing alcoholic extracts

Achillea santolina flowers were collected from around Mashhad and approved by the herbarium center of the Faculty of Basic Sciences of Islamic Azad University of Mashhad under herbarium number 1985 (Herbarium of Islamic Azad University of Mashhad IAUM). First, Achillea santolina flowers were dried and powdered in the shade, and then the alcoholic extract was prepared by the Soxhlet method in the plant research room of the Faculty of Sciences of Islamic Azad University, Mashhad branch. For this purpose, 30 grams of dry Achillea santolina flower

powder was poured into a special cartridge paper and placed in the machine, and 300 ccs of pure ethanol were used as a solvent. In the end, the solvent was removed from the alcoholic extract.

2.2 Design of groups

In this research, 24 white Wistar male rats weighing 200-250 gr were procured from the animal department of the Ferdowsi University of Mashhad, Faculty of Pharmacy. The rats were exposed to a standard 12/12 h light and dark cycle maintained at 22–24 °C and fed laboratory chow and water ad libitum and the experiment was approved by the Ethics Committee of the IAUM with code number 11130517902006. Animal research in this study was conducted to international guidelines and ethical regulations. Then, the animals were divided into 4 groups of 6, including Group A: Control, Group B: Compression, Group C: Compression + treatment with a dose of 50 mg/kg of alcohol extract, Group D: Compression + treatment with a dose of 75 mg/ kg of alcohol extract were divided and all of them except the control group underwent sciatic nerve compression. In the experimental groups, the extract was injected intraperitoneally two times at intervals of one week after compression of the sciatic nerve [21]. But the control group was injected with the physiological serum.

2.3 Animal model

Animals surgery was performed under deep anesthesia by intraperitoneal injection of Rompun (6 mg/kg) and ketamine (60 mg/kg) [21, 22]. Following anesthesia and hair removal, an incision of 2-3 cm was made in the right thigh of the animal, and the sciatic nerve was exposed by splitting the muscle at the depth of this area. The sciatic nerve was compressed for 60 seconds by using artery forceps. For all rats, the same method and forceps were used for compression. After compression, the nerve was placed in its normal place and the edges of the wound were sutured and the place was disinfected. In the treatment group, the first step of extract injection (50 and 75 mg/kg) was done immediately after the compression operation. The second step of extract injection in the treatment groups took place one week after the first injection.

2.4 Preparation of tissues

Next, 28 days after the compression, the animal tissues were partially stabilized using the perfusion method. To do this, after anesthesia, a triangular cut is made from the end of the sternum to expose the heart, then the catheter connected to the perfusion device is inserted into the aorta from the left ventricle, followed by an incision in the right atrium. To fix animal tissues, the blood in the veins was first washed with physiological serum, then the fixative (10% saline formalin) was injected into the general blood circulation [21, 22]. After that, the animal's lumbar spinal cord was sampled. The spinal cord was exited from the inside vertebral column up to the end of the terminal cone, then 8 mm long samples were prepared 18 mm above the end of the terminal cone of the spinal cord. Tissue samples were fixed in a 10% saline formalin solution for two weeks, and then the prepared tissues entered the tissue passage stages, which involve three steps: dehydration from the tissue, clarification with xylene, And the step of impregnation with paraffin. In the cutting stage with a microtome, 7-micron sections were prepared serially and 3 out of 30 slices were successively transferred to the slide and then the samples were stained with toluidine blue dye. Then, using a photomicroscope with a magnification of 200, photographs were taken of two Sequential sections on the right side of the slides prepared from the anterior horn region of the spinal cord. The physical dissector method was used to count the alpha motor neurons of the anterior horn of the spinal cord on the right side. In this method, neurons are counted in a reference frame. After counting the neurons, the neuronal density (ND) was calculated as follows [21-25]:

$$ND = \sum Q / \sum Frame \times V_{dissector}$$

$\sum Q$: The total number of neurons counted in a sample

$\sum Frame$: The total number of times sampled in a sample

$V_{disector}$: Volume of the sampling frame equal to $V_{disector} = H \times A_{Frame}$

A_{Frame}: sampling frame area (25 x 25 mm)

H: The distance between two sections or the thickness of two sections

2.6 Statistical analysis

After obtaining the ND, the data were analyzed using the Minitab13 software and the ANOVA

statistical test, and the significance level was considered less than 0.05 [23-25].

3. Results

As shown in Figure 1 and, Table 1 after 28 days, the average density of the number of neurons in the sham group shows a significant decrease compared to the control group (P <0.001). Also, the ND in the groups treated with alcohol extract (dose of 50 and 75 mg/kg of alcohol) showed a significant increase compared to the compression group, which this increase was higher in the group with a dose of 75 than other groups (P <0.001).

To verify the results of this research, images of alpha motoneurons of the anterior horn of the right half of the spinal cord were investigated, which are shown in Figure 2. In all the images, staining was done with the toluidine blue method at pH = 4.6. Figure A and B show, that after nerve compression, the nucleus of the neuron is pushed aside and is gradually disappearing, additionally, the neuron's shape is multifaceted rather than spherical. In the treatment groups (C and D) by injecting the extract, the nucleus of the neuron is visible again in the group with a dose of 75 mg/kg, the clarity of the nuclei is more visible and the shape of the neuron is closer to the control group.

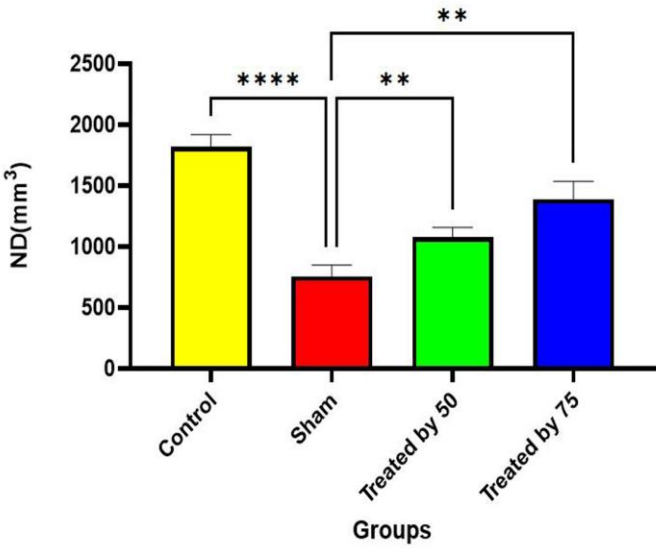

Figure1. Comparing the neuronal density (ND) between the Sham group and other groups. **: P <0.01, ****: P <0.0001

4. Discussion

According to the current study, the compression group's neuron density was lower than that of the control group. It is significant because it indicates that the compression of the animal's sciatic nerve caused the effects of central degeneration to manifest retrogradely toward the motor neuron cell bodies in the anterior horn of the spinal cord. Lastly, it shows that the neuronal density in the compression group was lower than in the control group. Additionally, compared to compression, the neuronal density rose in the treatment groups. Numerous biological processes take place at the cellular and molecular levels after sciatic nerve compression, including apoptosis, increased calcium entry into neurons, the release of excitatory neurotransmitters, including glutamate, generation of free radicals, and activation of inflammatory processes [3].

The reactive nitrogen species (RNS), such as nitric oxide (NO), are also one of the main factors in pathophysiologic response during ischemia. Reactive oxygen species (ROS), also known as programmed cell death, are produced by cells and lead to apoptosis. Due to some herbs' high antioxidant content, there is some evidence that they have neuroprotective properties [26]. Oxidative stress-relieving substances or those that boost mitochondrial defense against reactive oxygen species like superoxide and hydrogen peroxide will have neuroprotective effects [27]. In comparison to 66 extracts from desert plants examined in 2011 studies on the anti-inflammatory activities of Achillea fragrantissima, the extract from this plant was the most efficacious extract and blocked 70% of the NO generated by activated cells. This decrease was dose-dependent and wasn't brought on by the extract's cytotoxic effects. Additionally, Achillea fragrantissima extracts reduced NO and ROS generation from primary cultures of activated microglial cells and suppressed the expression of proinflammatory cytokines IL-1b and TNFa as well as proinflammatory enzymes COX-2, iNOS, and MMP-9 when LPS was present [28]. Borneol, one of Achillea's significant chemicals, is crucial in avoiding the peroxidation of cellular lipids [29]. The non-polar components of the achillea plant extract that are linked to camphor, 1,8 cineol, and Borneol contain the majority of the anti-inflammatory compounds. According to studies, Borneol lessens neuronal damage via multifunctional

Table 1. The density of neurons in each of the studied samples in different groups

Rat No.	Control group (n=6)	Compression group (n=6)	Treatment group (50 mg/kg alcohol) (n=6)	Treatment group (75 mg/kg alcohol) (n=6)
1	1946.653	761.7337	1184.919	1354.193
2	1819.697	931.0079	1057.964	1184.919
3	1862.016	677.0967	1015.645	1269.556
4	1862.016	761.7337	973.3265	1438.83
5	1777.379	719.4152	1142.601	1523.467
6	1650.423	677.0967	1100.282	1565.786
Mean ± SD	1819.697±100.14	754.68±94.31	1079.12±79.17	1389.46±147.61

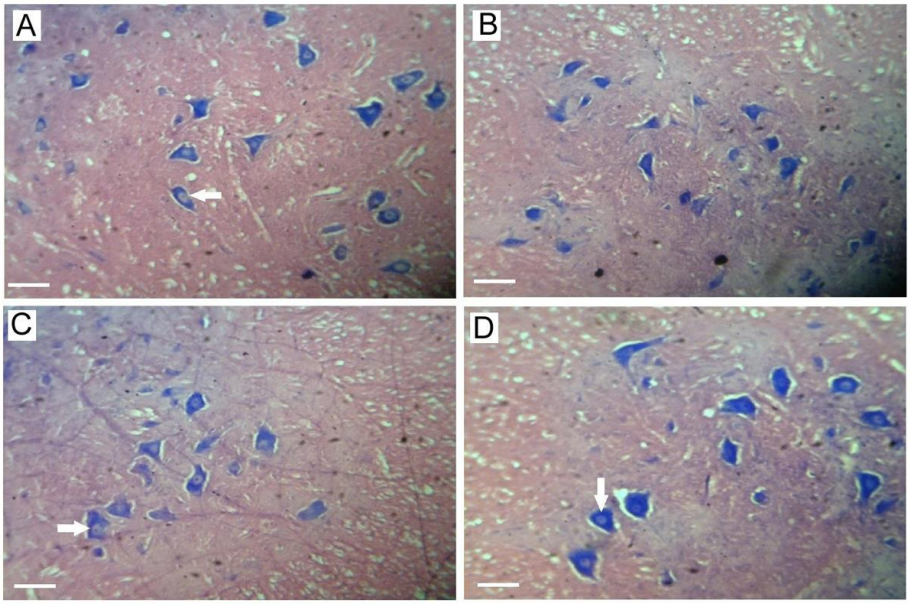

Figure 2. Comparing alpha motoneurons (white arrow) of the spinal cord in the studied groups showed by H&E staining: Control group (A), Sham group (B), Treatment group by 50mg/kg alcohol extract (C), Treatment group by 75mg/kg alcohol extract (D). Magnification: 1600x, Scale bar: 50µm.

signaling networks, neuronal nuclear density, and mitochondrial membrane dispersion. The suppression of apoptosis-related caspases, inhibition of IROS nuclear translocation, control of the NO/iNOS pathway, and intracellular reduction of inflammatory molecules are the mechanisms behind this reversal [30]. According to research by Alcaraz and colleagues, flavonoids, which all work as antioxidants and have anti-inflammatory characteristics, are abundant in the aerial sections of Achillea [31]. In a 2019 study by Etehadpour et al., it was revealed that this compound's proportion was lower and that of the borneol compound was higher than in previous research. These results generally demonstrated that the quality and amount of a plant's essential oil composition could change depending on the geographical setting [32]. The findings of Akif Açıkgöz study on antibacterial activity are shown. All of the bacteria, yeast, and fungi that were utilized demonstrated intense antibacterial activity against the volatile oils of Achillea gypsicola. The volatile oils of Achillea gypsicola were most effective against fungus and bacteria when obtained at the full blooming stage. Pre-flowering stage and post-flowering stage values were obtained for the volatile oil samples that were the most active. Each oil shows effectiveness against germs and fungus [33]. There is a significant difference between the compression group and the treatment groups receiving 50 mg/kg and 75 mg/kg of alcohol when comparing the density of the number of neurons. The injection of the alcoholic extracts of the aforementioned plant in two doses of 50 and 75 mg/kg will therefore enhance the density of the number of neurons and have neuroprotective effects, which are likely caused by the presence of alcohol in

soluble elements. This result was consistent with Alikhanzade and his colleagues' studies on the effects of 75 mg/kg of the hydroalcoholic extract of the Achillea plant on the treatment of inflammation in stomach ulcers [26]. The effects of ingesting this plant are therefore likely dose-dependent, based on the results of this study and the research that has been done in the area of its anti-inflammatory capabilities. Additionally, it appears that the Achillea plant's alcoholic extracts include one or more powerful compounds, such as flavonoids, that assist cells in surviving after axon damage by lowering inflammation, having antioxidant properties, or delaying neuronal death (anti-apoptosis).

5. Conclusion

According to the current research, an alcoholic extract of the Achillea santolina flower at various doses and injection times resulted in neuronal protection. The effects of this extract depended first on the dose and then on the amount of the active ingredient present in the alcoholic extract. More research will therefore be required in the future to complete the knowledge and efficient mechanisms, taking into account the potential use of the aforementioned plant's extract in the repair of nerve injury or inflammation.

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Authors' contributions

GhL, SR, NoA, SV, designed and performed all experiments. GhL and SH wrote the original manuscript. NaA and SV supervised the project and assisted in reviewing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical declarations

All procedures were performed in accordance with the guidelines of the Medical Ethics Committee of Islamic Azad University of Mashhad (Registered code: 11130517902006).

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