

The neuroprotective effect of alcoholic and aqueous extracts of *Achillea biebersteinii* leaves on spinal motoneurons denegation after sciatic nerve compression in rats

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ABSTRACT

Background and aims: Neurons are injured under physical, chemical and pathological conditions. Among high content of camphor and borneol, *Achillea biebersteinii* from Asteracea family may be effective in prevention the nervous injury progress. So, the aim of the present study was to evaluate the neuroprotective effect of *A. biebersteinii* leaves extracts on spinal motoneurons denegation after sciatic nerve compression in rats.

Methods: This study was carried out on forty eight male Wistar rats divided in eight groups including control (A), compression (B), compression and treated with 50 (C), 75 (D), 100 (E) mg/kg of alcoholic extract, compression and treated with 50 (F), 75 (G), 100 (H) mg/kg of aqueous extract of *A. biebersteinii*. In compression and experimental groups right sciatic nerve was highly compressed for 60 s. Extract was injected intraperitoneally in the first and second week after compression. After four weeks post-operative the lumbar segments of spinal cord were sampled, processed, sectioned and stained with toluidine blue (pH 4.65). Using stereological quantitative technique, the number of alpha motoneurons in the right horn of spinal cord were counted and compared with each other.

Results: According to the results, neuronal density showed significant decrease in compression group compared to control group ($P < 0.001$). Neuronal density in experimental groups C, D and E (50, 75, 100 mg/kg of alcoholic extract) as well as F, G and H (50, 75, 100 mg/kg of aqueous extract) was significantly ($P < 0.001$) more than that of compression group. Data analysis also showed that for both alcoholic and aqueous extract the concentration of 75 mg/kg had stronger activity than concentrations of 50 and 100 mg/kg.

Conclusion: The results showed that alcoholic and aqueous extracts of *A. biebersteinii* leaves have neuroprotective effects on spinal cord alpha motoneurons after injury.

Keywords: *Achillea biebersteinii*, Sciatic nerve, Degeneration, Neuroprotective.

INTRODUCTION

Nerve injuries are of important causes of permanent disability and have a strong negative effect on patients' quality of life. Following injury to the axon, the distal segment experiences Wallerian degeneration and loses its myelin sheath. The proximal

segment can either die by apoptosis or undergo the chromatolytic reaction.¹ It is estimated that nervous system injuries affect more than 90,000 people each year.² As a result of this high incidence of neurological damages, neuroregeneration is becoming a

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rapidly growing field devoted to the discovery of new methods to recover nerve functionality after injury. Nerve regeneration refers to the regrowth or repair of nervous tissues, cells or cell products that may include generation of new neurons, glia, axons, myelin, or synapses. Unlike the central nervous system, for the most part, the peripheral nervous system has an inherent ability for self-repair and regeneration. In clinical practice, surgical repair is usually required for treating nerve transection injuries, while drug administration is a possible therapy of choice for treating nerve crush injuries in order to promote nerve regeneration.¹⁻⁷ The results of the researches performed in the field of peripheral nervous system (PNS) regeneration indicate that the presence of cellular messenger molecules and neurotrophic factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), p27KIP1 protein, transforming growth factor beta (TGF- β), alpha lipoic acid (ALA) are effective in injury and regeneration process^{7, 8, 9, 10} In this regard, it seems that other natural stimulus such as *Achillea biebersteinii* (*millefolium*) can be used to reduce neurological sequela. Based on this, developing neuroprotective drugs to treat peripheral nerve injuries, including the therapeutic agents derived from natural medicinal plants, has become an active area of intense research. *Achillea biebersteinii*, known commonly as Yarrow, is a flowering

plant in the family Asteraceae that has a long history as a powerful 'healing herb' used topically for wounds, cuts and abrasions.¹¹ *A. biebersteinii* has several biological activities such as antiseptic, analgesic, anti-inflammatory, antimicrobial and wound healing properties. Several compounds of *Achillea* are including monoterpenes, sesquiterpenes, diterpenes, triterpenes, flavonoids, phenolic acids, coumarins, lignans and caffeolquinic acid derivatives.¹²

Generally, the main compounds exist in *A. biebersteinii* leaves are cineole, borneol and camphor.¹³ which have neuroprotection and anti-apoptotic properties.¹⁴ Moreover, Due to having tannin compounds and aromatic bitter substances, *A. biebersteinii* is useful for nervous system, heart (weak muscles), general weakness treatment, and cardiomyopathy, nervous system diseases such as neurasthenia, hysteria and epilepsy.¹⁵ Therefore, the main purpose of this research was to evaluate the effect of aqueous and alcoholic extracts of Yarrow leaves to prevent or delay the progression of spinal motor neuron degeneration after rat sciatic nerve compression and consequently promoting the regeneration process.

METHODS

A. biebersteinii leaves were supplied commercially and confirmed by Dr Jafari at the herbarium centre of Islamic Azad University of Mashhad (IAUM) with herbarium number 9058.

A. biebersteinii leaves were completely ground using a grinder. Alcoholic and aqueous extracts of *A. biebersteinii* leaves were prepared using soxhlet extractor (model H626). First 50 grams drained leaves was put into filter paper and placed in the extractor. Respectively, 450 cc of ethanol and distilled water were used for preparation of alcoholic and aqueous extracts of *A. biebersteinii* leaves. Finally, solvents were removed from both extracts.¹⁶

Forty eight male Wistar rats weighting 200-250 g received from Razi Serum and Vaccine Research Institute, Mashhad, Iran were used in this study. The rats were held in department of biology, Islamic Azad University of Mashhad at 21 °C, humidity of 50%, and a cycle of 12 hours light- dark. Moreover, all rats had access to sufficient food and water. All procedures were in accordance with the local guidelines for the care and use of laboratory animals and were approved by the Islamic Azad University Mashhad branch (2013).

Male Wistar rats were allotted into 8 groups (6 rats per group): A (control or sham): For baseline measurement in this group on the right hind limb an operation was performed which exposed the sciatic nerve without compression. B (Compression group): In this group after operation the right hind limb sciatic nerve was compressed. C (compression + injection of 50 mg/kg alcoholic extract of *A. biebersteinii* leaves), D (compression + injection of 75 mg/kg alcoholic extract of

A. biebersteinii leaves), E (compression + injection of 100 mg/kg alcoholic extract of *A. biebersteinii* leaves), F (compression + injection of 50 mg/kg aqueous extract of *A. biebersteinii* leaves), G (compression + injection of 75 mg/kg aqueous extract of *A. biebersteinii* leaves), H (compression + injection of 100 mg/kg aqueous extract of *A. biebersteinii* leaves). These animals were compressed and simultaneously extracts was injected i.p., 2 times.¹⁷

The rats were anesthetized under intraperitoneal injection of 60 mg/kg Rompun and 6 mg/kg Ketamine.¹⁶ After removing animal hair on right femur, skin was cut for 2-3 cm and femoral muscle underwent surgery in order to find sciatic nerve. In the next stage, compression of sciatic nerve was done for 60 seconds using locker pincers (second lock). After compression, the injured part was sterilized and stitched.

In experimental groups the first injection of extract was done immediately after compression. After consciousness, the rats were transferred into separate cages and kept under standard conditions. Second injection of extract was done one week after the first injection. After 28 days of compression, animals' tissues were fixed using perfusion method and then sampling was performed from lumbar spinal cord.¹⁷ Spinal cord was taken out of spinal column to cone medullary end and then after going 18 mm up cone medullary, 8 mm samples were provided. Samples were kept in fixator

for two weeks and then entered tissue passage which included three steps: dehydration of tissue (using alcohol), transparency (using xylene), and soaking in paraffin. Cutting was carried out with microtome set, so that 7 micron cuts were transferred to glass slides. The work continued until 30 slides were prepared. The slides were then stained with toluidine blue.¹⁸ In the next step, some photos were taken from spinal anterior horn in the right part of glass slides. Dissector method was used to count alpha motor neurons of right anterior horn. In this method neurons are counted in a reference framework. When a particle is in the reference framework but is not present in the next frame (next consecutive cut), it is counted. However, if a neuron is present in both frameworks, it is not counted.^{17,18} Neuron density was estimated as below:

$$ND = \frac{\Sigma Q}{\Sigma \text{ frame}} \times V \text{ dissector}$$

In which: ΣQ : total neurons counted in one sample

Σ frame: total number of sampled cases in a sample

V dissector: volume of sampling frame which equals:

$$V \text{ dissector} = A \text{ frame} \times H$$

A frame: the area of sampling frame

H: distance between two consecutive slices, or depth of each cut.

Data were analysed using Minitab 13 software, Tukey and ANOVA statistical tests (for binary comparison). Tests significance level was considered $P < 0.05$.

RESULTS

In this study, the neuroprotective effect of alcoholic and aqueous extracts of *A. biebersteinii* leaves were individually investigated on spinal motoneurons denegation after sciatic nerve compression in rats. The result of comparing density of alpha motor neurons in spinal anterior horn between group A (control) and B (compression) was explained. The number of alpha motor neurons in A and B (compression) groups was respectively 1771 ± 73 and 671 ± 79 (mean \pm standard deviation) and this difference in neuron density of these two groups was significant ($P < 0.001$).

The average density of alpha motor neurons was respectively 1044 ± 81 , 1278 ± 20 and 1130 ± 115 in experimental groups C, D and E (respectively, 50, 75, 100 mg/kg of alcoholic extract). Significant difference ($P < 0.005$) was shown when the density of alpha motor neurons in these three groups (C, D, E) and compression group (B) was compared.

Average density of alpha motor neurons was respectively $1052 + 105$, $1344 + 76$ and $1153 + 110$ in treatment groups F, G and H. Each group showed a significant difference with compression group ($P < 0.001$).

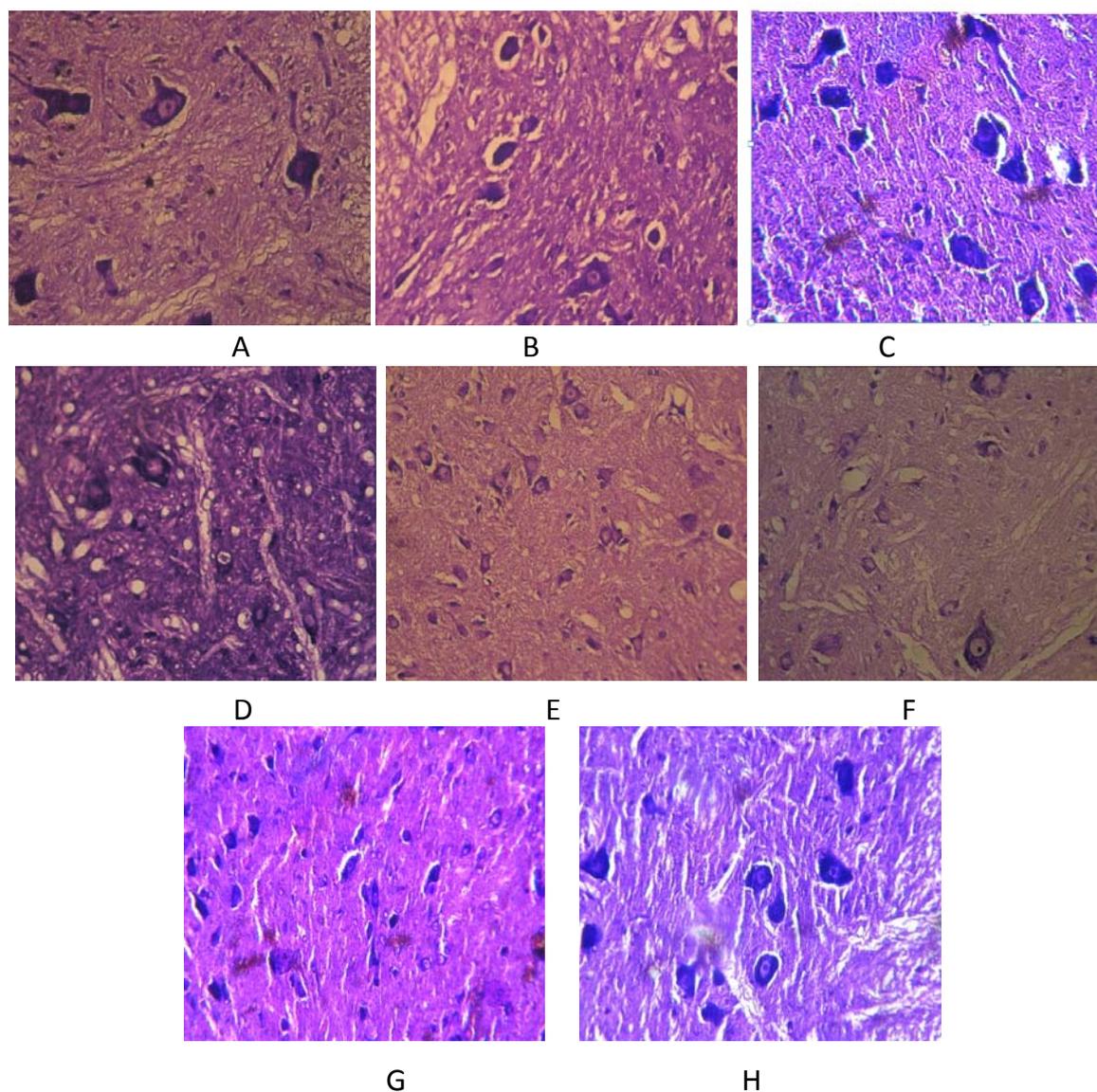


Fig.1: Cross-sectional representation of spinal and alpha motor neurons of spinal anterior horn in the right part of different groups (toluidine blue staining, zoom X 400)

A: control, B: compression, C: compression + treatment with a dose of 50 mg/kg alcoholic extract, D: compression + treatment with a dose of 75 mg/kg alcoholic extract, E: compression + treatment with a dose of 100 mg/kg alcoholic extract, and F: compression + treatment with a dose of 50 mg/kg aqueous extract, G: compression + treatment with a dose of 75 mg/kg aqueous extract, H: compression + treatment with a dose of 100 mg/kg aqueous extract.

DISCUSSION

During neuronal damage, neurologic diseases, and ischemia, a large number of free radicals and inflammatory cytokines are

released due to oxidative stress reactions. The reactive nitrogen species (RNS) such as nitric oxide (NO) are also one of the main

factors in pathophysiologic response during ischemia. The production of reactive oxygen species (ROS) in cells causes apoptosis or programmed cell death. There are some evidences about the neuroprotective effect of some herbs due to their high content of antioxidant components. Several compounds of *Achillea* are including monoterpenes, sesquiterpenes, diterpenes, triterpenes, flavonoids, phenolic acids, coumarins, lignans and caffeolquinic acid derivatives.¹² Generally, the main compounds exist in the leaves are cineole, borneol and camphor.¹³ Borneol as one of the important compounds of *Achillea Millefolium* plays a major role in neuroprotection and prevention of apoptosis and lipid peroxidation.^{14, 19}

The result of the present study showed that neuronal density in the compression group (B) was significantly lower than that of control group (A) ($P < 0.001$). It is said that sciatic nerve compression caused central degeneration effects retrograde towards the cell body of motor neurons in anterior horn of the spinal cord. The injuries to the nerve fibre in the distal part of neurons caused the Wallerian degeneration along with calcium influx and histological changes such as axonal fragmentation and myelin disruption.¹ The apoptosis along with denervation which is an example of neuronal cell death due to the structural changes can lead in neuronal death.^{20, 21}

Furthermore, neuronal density in all experimental groups (compression + alcoholic or aqueous extract) was increased more than the density in the compression group ($P < 0.01$). Studies indicated that borneol could reduce neuronal damage caused by OGD/R through scattering the mitochondrial membrane, condensing neuronal nuclei and multi-functional signaling pathways. The reverse mechanism is in reducing intracellular ROS, adjusting iNOS/NO pathway, reducing the release of inflammatory factors and blocking nuclear

translocation of NF-KBP65 and stagnating caspases associated with apoptosis.²² The neuroprotective effects of borneol firstly are because borneol decrease ROS production; secondly inhibit the NF-KB activity, subsequently inhibit the iNOS/NO pathway, and decrease TNF α ; and thirdly protect the mitochondria and subsequently caspase family associated with apoptotic signaling pathway. Therefore, the inhibition of NFKB and IKB, and transduction of signaling pathways are probably the main neuroprotective role of borneol.¹⁴ On the other hand, anti-inflammatory factors of the extract of *A. millefolium* are located mainly in the non-polar compounds that relates to borneol, 1, 8 cineol and camphor components.²⁷ The systemic treatment with 1, 8 cineol is effective in prevention of the inflammatory process.²⁴

This component represents an anti-inflammatory effect by reducing mitogenic cytokines such as interferon gamma produced in the mast cells in vitro.²⁵ In another study conducted on paw edema test and ulcerative colitis (a form of inflammatory bowel disease) induced by TBNS in rats and asthma patients, it became clear that this component inhibited the inflammation by inhibiting leukotrienes B₈, prostaglandin E₂, TNF α , IL-1 β , and thromboxane B₂.^{26, 27} It seems that the aqueous extract of Yarrow leaves contains effective substances such as flavonoids that help axons to regenerate after injury through reducing inflammation, preventing neuronal death (anti-apoptotic) or using antioxidant effects.

By comparison the neuronal density in compression group with 50, 75 and 100 mg/kg alcoholic treatment groups, a significant difference ($P < 0.001$) was observed. Therefore, it can be concluded that the alcoholic extract injection in three different doses: 50, 75, 100 mg/kg increases the number of neurons and therefore

presented neuroprotective effects that result from the presence of dissolved components in alcohol such as borneol. Moreover, the research of Alcaraz *et al* suggested that the aerial parts of *Achillea millefolium* are rich in flavonoids that act as an antioxidant and have anti-inflammatory effects. In this regard, the flavonoids that exist in this plant can justify anti-inflammatory effects by producing antioxidant and anti-inflammatory effects. In this case, it was found that such flavonoids are capable in inhibiting enzymes involved in the production of oxygen free radicals such as cyclooxygenase (COX), lipoxygenases, microsomal monooxygenase and glutathione s-transferase. Another anti-inflammatory effect of flavonoids is to regulate the loss in NO production and inhibit neutrophils degranulation so that it reduces the activity of inflammation-derived enzymes.¹⁴ For instance, isoornithine inhibits the activity of xanthine oxidase and has an anti-rheumatoid arthritis (RA) and anti- acute gouty arthritis effects. Furthermore, the plant's existing flavonoids regulate the arachidonic acid metabolism.²⁸

By comparing data resulted from 50, 75 and 100 mg/kg alcoholic treatment groups as well as 50, 75, 100 mg/kg aqueous treatment groups, it is noted that neuroprotective effects at the 75 mg/kg dose is more than 50 and 100 mg/kg doses. Therefore, concerning the mentioned data and by comparing the studies conducted in the field of the plant's anti-inflammatory effects, these effects are probably dose-dependent. Based on the studies of Zarin Ghulam *et al.* on anti-inflammatory role of defatted methanol extract of the aerial parts of the yellow Yarrow plant, the 75 mg/kg dose had the most anti-inflammatory effects.^{23, 26} These results are closely in accordance with the studies of Rashidi *et al.* who reported the greater impact of 75 mg/kg hydroalcoholic

extract of Yarrow plant on stomach ulcers.¹⁴ Furthermore, with a regard to the researches performed with 25, 50, 75 and 100 mg/kg hydroalcoholic extract of the plan on anti-inflammatory effects of Yarrow leaves, the neuroprotective effects are probably dose-dependent so that the 75 mg/kg dose had the most neuroprotective effects and using higher doses can cause allergic reactions which is mentioned in the studies of Rashidi *et al.*²⁹ In addition, based on Arzi *et al.* 75 mg/kg hydroalcoholic extract of the plan had the most anti-nociceptive effects, probably because of the most-effective components such as water-soluble flavonoid glycosides. In 1969, Goldberg *et al.* suggested that the aqueous extract containing proteins and carbohydrates could reduce inflammation by 25 percent.²⁸

All photos showed that in experimental groups the neurons have very normal shape compared with compression group. After compression nucleus was lead to one side and be despaired little by little. Also the shape of neuron is changed to multi-level. In treatment groups, injection extracts increased the neural density and the nucleus was appeared again.

CONCLUSION

The injections of different concentrations of aqueous and alcoholic extract of yarrow leaves resulted in neuroprotection activity in vivo. Furthermore, regarding the dose-dependent effects of the studied extracts, 75 mg/kg dose is considered the most effective one. Due to possible applications of the plant extract in neurologic sequelae (neurological injury) and inflammation recovery process, further research is needed to provide more effective approaches and information.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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