

THE EFFECT OF CANNABIS SATIVA LEAVES AQUATIC EXTRACT ON NEUROGLIA DENSITY AFTER SCIATIC NERVE INJURY IN RATS

Maryam Tehranipour* and Bibi Zahra Javadmoosavi

Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran

Summary

Proliferation of glial cells is one of the hallmarks of CNS responses to neural injury. These responses are likely to play important roles in neuronal survival and functional recovery after central or peripheral injury. *Cannabis sativa* has antioxidant effect. The aim of this study is carried out to examine the effects of aquatic extract of *Cannabis sativa* leaves on neuroglia density after sciatic nerve injury in rats.

Thirty two adult male wistar rats were used and divided to four groups (control, compression and two experimental groups). In compression and experimental groups right sciatic nerve were highly compressed for 60 s, assigned to experimental groups (Compression + aquatic extract of *Cannabis sativa* injections ($25, 50 \text{ mgkg}^{-1}$, ip, 2 time) (N=8).

After 4 weeks post-operative the lumbar segments of spinal cord were sampled, processed, sectioned serially and stained with toluidine blue (pH 4.65). By using stereological quantitative technique, the number of neuroglia in the right horn of spinal cord were counted and compared with each other. Statistical analyses showed remarkable increase in the number of neuroglia in the compression and decreased in experimental group with doses (25 mgkg^{-1})($p < 0.05$). It shows that *Cannabis sativa* leaves aquatic extract has Antiinflammation effect in low doses.

Key words: Cannabis sativa, neuroglia density, sciatic nerve

Running title: Cannabis sativa aquatic extract on neuroglia density

Corresponding Author: Tehranipour Maryam, Department of Biology, Mashhad Branch, Islamic Azad University, , Mashhad, Iran. Tel: +98511835050 Fax: +985118424020. E mail: maryam_tehranipour@mshdiau.ac.ir

Introduction

Microglial and astroglial cells undergo prompt responses to peripheral motor and sensory axon injury. These responses include proliferation of microglial cells as well as hypertrophy and increased levels of glial fibrillary acidic protein around the axotomized motoneurons and in the central projection territories of peripherally axotomized sensory ganglion cells(1). Proliferating microglial cells migrate towards reacting motoneurons, however, without directly apposing their cell membrane.

Astroglial cells, on the other hand, increase their structural interrelationship with reacting motoneurons, seemingly at the expense of some presynaptic terminals. In sensory projection areas, microglial cells phagocytose degenerating axons and terminals. Beyond these observations, the functional role of the central glial cell response to peripheral nerve injury is obscure (2). New research implicates spinal cord glia as key players in the creation and maintenance of pathological pain(3).

The inflammation conditions to produce cell death in both the central nervous system and the peripheral nervous system is well established with examples involving all cell types readily citable(4). In contrast to the varied nature of primary initiating events responsible for cell death, there is a smaller group of prevalent secondary processes such as excitotoxicity, oxidative stress, energy deprivation, and dysregulation of Ca^{2+} homeostasis that commonly interact to bring about a cell's demise and determine which death pathway is followed. For both neurons and glial cells, there are two well-recognized pathways leading to cell death: apoptosis and necrosis. Individual cells within a given population may follow either pathway and it is possible for some cells to exhibit characteristics inherent to both apoptosis and necrosis. However, there are sufficiently established biochemical and morphological characteristics to distinguish the prevailing mechanisms of each of these pathways(5).

Proliferation of glial cells is one of the hallmarks of CNS responses to neural injury. These responses are likely to play important roles in neuronal survival and functional recovery after central or peripheral injury. The positive microglial cells respond to the sciatic nerve injury becoming activated and macrophage and express MHCII molecules (6). Such activated microglia apparently may serve as neurosupportive cells, providing neuroprotection and scavenging cellular debris in response to the injury (7). Virtually all CNS injuries trigger a rapid activation of microglial cells. Once activated, microglial cells present a robust antigenic profile. They participate in the pathogenesis of neurological disorders by secreting various inflammatory molecules such as cytokines or nitric oxide. When CNS cells die, microglia can be further activated and become phagocytes. While considerably different in nature, all of these morphological and physiological properties have been used to identify microglial cells(8).

Cannabis is an annual, dioeciously, flowering herb. The leaves are palmate compound or digit ate, with serrate leaflets. *Cannabis* normally has imperfect flowers, with staminate "male" and pistil late "female" flowers occurring on separate plants (9).

Cannabis plants produce a unique family of terpeno-phenolic compounds called cannabinoids, which produce the "high" one experiences from smoking marijuana. The two cannabinoids usually produced in greatest abundance are cannabidiol (CBD) and/or Δ^9 -tetrahydrocannabinol (THC), but only THC is psychoactive (10). A synthetic form of the main psychoactive cannabinoid in *Cannabis*, Δ^9 -tetrahydrocannabinol (THC), is used as a treatment for a wide range of medical conditions(11).

In the United States there has been considerable interest in its use for the treatment of a number of conditions, including glaucoma, AIDS wasting, neuropathic pain, treatment of spasticity associated with multiple sclerosis, and chemotherapy-induced nausea," the agency has not approved "medical marijuana". Clinical trials have shown the efficacy of cannabis as a treatment for cancer and AIDS patient (5).

Potential therapeutic uses of cannabinoid receptor agonists include the management of spasticity and tremor in multiple sclerosis/spinal cord injury, pain, inflammatory disorders, glaucoma, bronchial asthma, cancer, and vasodilatation that accompanies advanced cirrhosis. CB(1) receptor antagonists have therapeutic potential in Parkinson's disease(12).

Then according to antioxidant effect of this plant this study aimed to evaluate the effect of *Cannabis sativa* aquatic extract on neuroglia density after sciatic nerve injury in rat.

Material and Methods

The *Cannabis sativa* (herbarium code 2521) was supplied by Islamic Azad University of Mashhad, Iran (2010).

Animal subjects

Thirty two male, Wistar rats weighting between 300-350 g served as subjects for these experiments. All animals were housed individually and maintained on a 12/12 light/dark cycle, with lights on at 6.00h. Ambient temperature in the animal facility was kept at $22\pm 2^{\circ}\text{C}$. Food and water was given ad libitum.

Extraction

Cannabis sativa was collected from a reign around mashhad and was coded with Islamic Azad University of Mashhad, Iran herbarium. For extraction 50g powder pod with 300 cc wather were mixed and extraction perform with Soxhlet apparatus (13). After obtaining extract, it was situated in oven with temperature ($45^{\circ}\pm 2^{\circ}$) for 48 hours to remove solvent.

Groups

Thirty two rats divided into four groups:1) Control; 2)Compression ; 3)Compression + aquatic extract of *Cannabis sativa* injections (25mgkg⁻¹ , ip ,2 time); 4) Compression + aquatic extract of *Cannabis sativa* injections (50mgkg⁻¹ , ip ,2 time) (N=8).

Surgery

Animals were anesthetized under intera peritoneal injection of a mixture of ketamin and xylazine (6,66mgkg⁻¹).

Right sciatic nerve was exposed through a gluteal muscle splitting incision. At this location the nerve trunk was crushed for 60 seconds period between prongs of #5clamp forceps. The muscle and skin were then closed with 14mm stainless steel sutures (14).They could consume enough water and specified food during the experiment. In care groups, the extract injection was carried out immediately after compression during 28 days (Each week one injection).

After 28 day following perfusion a block of the spinal cord segments L4 toL6 (approximately 8mm length) was removed while sciatic nerve roots of both sides were still attached it. Since the nervous tissues are very sensitive and autolysis rapidly. Besides fixators cannot penetrate in spinal cord because of though cover around it. So for better fixation, perfusion method was used. When perfusion finished, sampling of spinal cord was began. The spinal cord was completely separated and 8mm samples at

the injured area (L4-L6) entered to passage stage, then entered to cutting stage and serially 7 Mm sections were prepared and colored with toluidine blue. Required photos from front horn of spinal cord for future studies were taken according their numbers. Two photos were taken from two serial sections, one of anterior horn right half of first section and another from anterior horn right half of second section. The magnitude of microscope in this stage was $5 \times 10 \times 2/5 = 100$.

In order to count neuroglia cells random systematic method was used and dissector method was used for counting particles (15).

Statistical analysis

The ratio of numerical density of neuroglia cells in samples of spinal cord was used as an index of neuroglia cells death. All quantitative data were analyzed using ANOVA and t-test.

All data were presented as mean \pm S.E. Differences were considered statistically significant when ($p < 0.05$).

Results

The results indicate several facts:

1-The number of neuroglia cells increases in compression group in compare with control group ($p < 0.05$) (Fig.1).

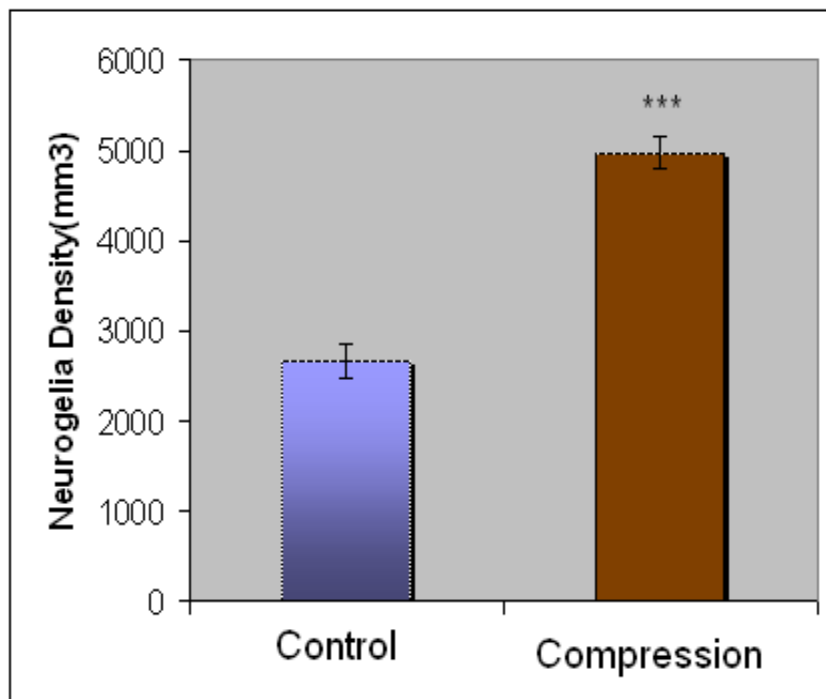


Fig.1. Compression of the neuroglia cells density in compression and control groups ($p < 0.05$). Data are presented as mean \pm S.E. ($n = 8$ in each group).

2- The neuroglia cells density (number of neuroglia cells) in experimental (25mgkg⁻¹) group is decreased in compare with compression group ($p < 0.05$) (Table.1) (Fig.2).

Table.1: Neuroglia cells density in anterior horn of spinal cord in different groups. Data are presented as mean± S.E. (n=8 in each group).

Groups	NV (Mean ±S.E.)
Control	2669 ±192
Compression	4976 ±180
Treatment (25mgkg-1)	4344 ± 98
Treatment (50mgkg-1)	6761 ± 138

3- The neuroglia cells density (number of neuroglia cells) in experimental (50mgkg-1) group is increased in compare with compression group ($p < 0.05$) (Table.1) (Fig.2).

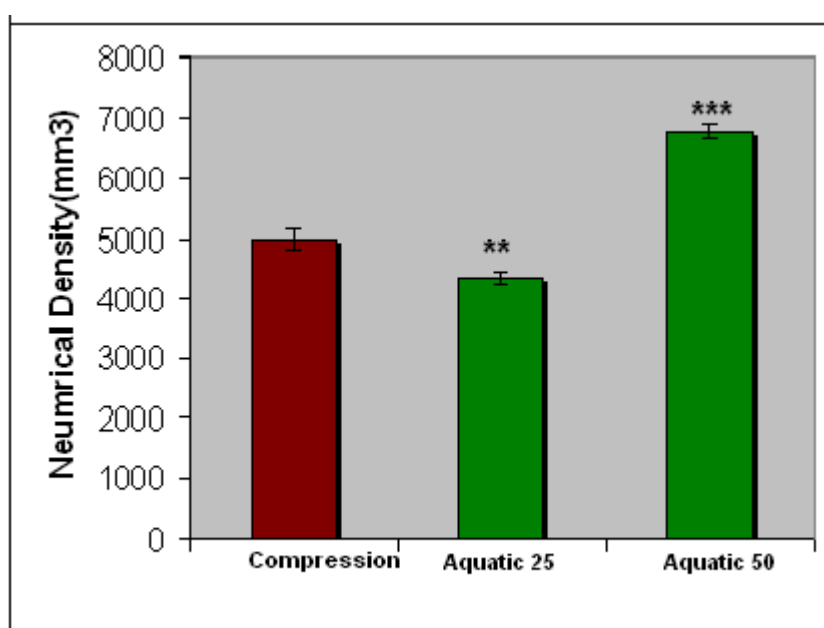


Fig.2: Comparison of Neuroglia cells density in anterior horn of spinal cord in two different dosages (25, 50 mg/kg) with compression group.. Data are presented as mean± S.E. (n=8 in each group).

4-The size and number of Neuroglia cells in experimental (25mgkg-1) groups have remarkable decrease in compare with compression group (Fig.3).

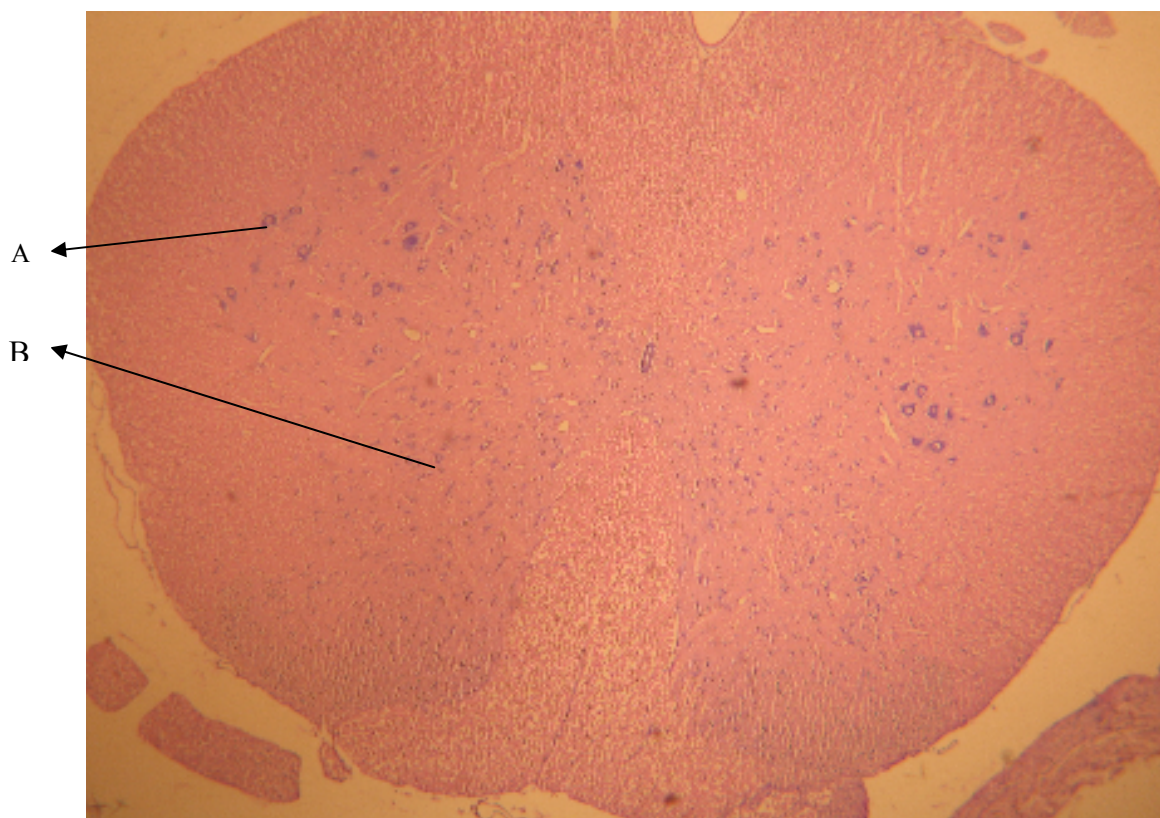


Fig.3: The cross section of spinal cord anterior horn (magnitude 200x And painting in toluidin blue)A: Alpha motoneuron B: Neuroglia cell

Discussion

Following peripheral nerve injury perineuronal satellite cell reaction in the corresponding spinal ganglion is observed. The mechanisms underlying the glial responses to axon injury remain unknown. It is possible that *cannabis sativa* aquatic extract has protective effects on injured central nervous system cell and the results of this research show this hypothesis. It was shown that the neuroglia density in compression group increased in compare with control group (Table.1). But in treatment group with aquatic extract doses (25mgkg⁻¹) the neuroglia density was decreased ($p < 0.05$) in compare with compression group (Fig.2).

It may be concluded that there is some component in aquatic extract of *cannabis sativa* that protected neuroglia from proliferation after sciatic nerve injury or may be this component has had some roles in regeneration phenomena and act as a neurotrophic factors (Fig.3).

These factors consist of fibroblastic growth factors secreting from Schwann cells and macrophages that their synthesis is stimulated by cytokines and sticky molecules (16). Other neurotrophic factors affecting on correction are growth factor (NGF), growth factor originating from brain (BDNF), insulin – like growth factor (IGF) of integrin, laminin, collagen and fibronectin (17).

Following sciatic nerve injury, the generation of free radicals causes apoptosis in the cell body of spinal cord neurons (18). Antioxidants inactivate free radicals and prevent apoptosis in spinal cord neurons. Different species of *cannabis sativa* have anti oxidant effects and are responsible for collection and removing free radicals (19). Therefore protective effects of aquatic extract are due to its anti- oxidant role.

Although the aquatic extract with doses (25mgkg⁻¹) has antifalammation role but increasing the dose correlated with increasing the numerical density in nourogelia cell. As in treatment group with (50mgkg⁻¹) doses nourogelia density meaningful increased (Fig.2).

Cannabis sativa (marijuana) have been used both medicinally and recreationally for many centuries. Recent advances in the knowledge of its pharmacological and chemical properties in the organism, mainly due to Delta (4)-tetrahydrocannabinol, and the physiological roles played by the endocannabinoids have opened up new strategies in the treatment of neurological and psychiatric diseases (12).

There are some evidences supporting the hypothesis that some herbs may also exert neurotrophic and inflammation actions (5). It provides neuronal differentiation and increase in neuritis outgrowth. This plants act by prevention of relieving glutamate (8).

Causing any pressure to axon or occurrence of axonic defeat induced changes in both distal and proximal parts because of breaking the connection of axon with neuron's body (20). In distal part, both axon and myelin cover is degenerated completely. In this defeat, emerging 2-3 days later, Endoneurim layers keep unchanged. Considering the ability of producing new myelin, Schwann cells increase along degenerated fiber, so in distal part of defeated nerve, for myelin making in new branches of proximal part of nerve new circumstances is required (21).

In these phenomena neuralgias proliferated and act as macrophage cells (22). In addition increasing of inflammation proceeding is coordinated with proliferated of neuroglia cells. Then if some component could inhibit these phenomena, degeneration of central nervous system was decreased.

Evidence has accumulated over the last few years suggesting that endocannabinoid-based drugs may potentially be useful to reduce the effects of neurodegeneration. In fact, exogenous and endogenous cannabinoids were shown to exert neuroprotection in a variety of in vitro and in vivo models of neuronal injury via different mechanisms, such as prevention of excitotoxicity by cannabinoid CB₁-mediated inhibition of glutamatergic transmission, reduction of calcium influx, anti-oxidant activity, activation of the phosphatidylinositol 3-kinase/protein kinase B pathway, induction of phosphorylation of extracellular regulated kinases and the expression of transcription factors and neurotrophins(23), lowering of cerebrovasoconstriction and induction of hypothermia. The release of endocannabinoids during neuronal injury may constitute a protective response (24, 25, 26).

In total administration of *cannabis sativa* aquatic extract in low doses after sciatic nerve injury can decrease neuroglia density in compare with compression groups and have had anti inflammation effect.

Acknowledgment

This study was supported by Islamic Azad University of Mashhad, Iran. We thank Dr. Khayatade for her helpful comments and discussions.

References

1. Chaisuksunt V, Zhang Y, Anderson PN, Campbell G, Vaudano E, Schachner M, Lieberman AR. Axonal regeneration from CNS neurons in the cerebellum and

brainstem of adult rats: correlation with the patterns of expression and distribution of messenger RNAs for L1, CHL1, c-jun and growth-associated protein-43. *Neuroscience*(2000);100(1):87-108.

2. Anderson PN, Campbell G, Zhang Y, Lieberman AR. Cellular and molecular correlates of the regeneration of adult mammalian CNS axons into peripheral nerve grafts. *Prog Brain Res*(1998);117:211-32

3. King SA . Cannabinoids and Pain. *Psychiatric Times* (2008);25 (2): 56-59.

4. Gordon T. The physiology of neural injury and regeneration: The role of neurotrophic factors. *J Commun Disord*(2010);43(4):265-73.

5. Perry N, Court G, Bidet N, Court J, Perry E .European herbs with cholinergic activities: potential in dementia therapy. *Int J Geriatr Psychiatry* (1996);11:1063-1069.

6. Patro N, Nagayach A, Patro IK. Iba1 expressing microglia in the dorsal root ganglia become activated following peripheral nerve injury in rats. *Indian J Exp Biol*(2010);48(2):110-6.

7. Liu L, Rudin M, Kozlova EN. Glial cell proliferation in the spinal cord after dorsal rhizotomy or sciatic nerve transection in the adult rat. *Exp Brain Res* (2000);131(1):64-73.

8. Maier IC, Schwab ME. Sprouting, regeneration and circuit formation in the injured spinal cord: factors and activity. *Philos Trans R Soc Lond B Biol Sci*(2006);29:361(1473):1611-34.

9 . Lebel-Hardenack S, Grant S R. Genetics of sex determination in flowering plants. *Trends in Plant Science* (1997); 2(4): 130–136.

10. Small E, Beckstead HD. Common cannabinoid phenotypes in 350 stocks of *Cannabis*. *Lloydia* (1973);36: 144–165.

11. Costa B. "On the pharmacological properties of Delta9-tetrahydrocannabinol (THC)". *Chemistry & Biodiversity*(2007);4 (8): 1664–77.

12. Alsasua del val A . Implication of cannabinoids in neurological disease .*Cellular and molecular neurobiology*(2006);26:571-591.

13. Cicchetti E, Chaintreau A. Comparison of extraction techniques and modeling of accelerated solvent extraction for the authentication of natural vanilla flavors.*J Sep Sci* (2009);32(11):1957-64.

14. Behnam-rasouli M, Nikravesh M, Mahdavi M, Tehranipour M. Post-operative time effects after sciatic nerve crush on the number of alpha motoneurons, using a stereological counting method(Disector).*Iranian biomedical journal*(2000);4(1):45-49.

15. Tehranipour M, Kabiri M. The effect of exogenous testosterone administration on peripheral nerves regeneration after sciatic nerve compression in Rat. *J.B.S.*(2009)9(7):692-696.
16. Zhang Y, Roslan R, Lang D, Schachner M, Lieberman AR, Anderson PN. Expression of CHL1 and L1 by neurons and glia following sciatic nerve and dorsal root injury. *Mol Cell Neurosci*(2000);16(1):71-86.
17. Imaizumi, T., Lankford, K.L. and Kocsis, J.D. Transplantation of olfactory ensheathing cells or Schwann cells restores rapid and secure conduction across the transected spinal cord. *Brain Res*(2000);854(1-2),70-8.
18. Siniscalco D, Fuccio C, Giordano C, Ferraraccio F, Palazzo E, Luongo L, Rossi F, Roth KA, Maione S, de Novellis V. Role of reactive oxygen species and spinal cord apoptotic genes in the development of neuropathic pain. *Pharmacol Res* (2007);55(2):158-66
19. Hampson A J, Grimaldi M, Axelrod J, Wink D, Rosenthal R. Neuroprotective antioxidants from marijuana. *Annals of the New York Academy of Sciences*(2000):274-282:0077-8923.
20. Sakurai M, Nagata T, Abe K, Horinouchi T, Itoyama Y, Tabayashi K. Survival and death-promoting events after transient spinal cord ischemia in rabbits: induction of Akt and caspase3 in motor neurons. *J Thorac Cardiovasc Surg* (2003);125(2):370-7.
21. Rosenfield J, Paksima N. Peripheral nerve injuries and repair in the upper extremity. *Bull Hosp Jt Dis* (2001);60(3-4):155-61.
22. Hu P, McLachlan EM. Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. *Neuroscience*(2002);112(1):23-38.
23. Vanelderen P, Rouwette T, Kozicz T, Roubos E, Van Zundert J, Heylen R, Vissers K. The role of brain-derived neurotrophic factor in different animal models of neuropathic pain. *Eur J Pain.* (2010);14(5):473.e1-9.
24. Schomacher M, Muller HD, Sommer C, Schwab S, Schabitz WR. Endocannabinoids mediates neuroprotection after transient focal cerebral ischemia. *Brain research* (2008).1240:213-220.
25. Mechoulam R, Shohami E. Endocannabinoids and traumatic brain injury. *Mol Neurobiol*(2007);36: 68-74.
26. Grundy RI, Rabuffetti M, Beltramo M. Cannabinoids and neuroprotection. *Molecular neurobiology.*(2001).24(1-3):29-25