Influence of *Smyrnium cordifolium Boiss* extract on the hippocampus Mu-opioid receptors in Mice

Pari Nazari[1], Parichehreh Yaghmaei[1*], Alireza Rangin[2], Naser Abbasi[3]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Biology, Ilam Branch, Islamic Azad University, Ilam, Iran
3. Department of Pharmacology, Ilam University of Medical Sciences, Ilam, Iran

***Corresponding author:**Tel: +98 9122010222 Fax: +98
Address: Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
E-mail: Yaghmaei_p@yahoo.com
Received; 1/03/2020 revised; 26/05/2020 accepted; 9/06/2020

Abstract

Introduction: Agonists of the Mu-opioid receptors (MOR), such as morphine are lengthily used for the treatment of moderate to pain, depression and anxiety. But the dose involved achieving adequate pain relief often elicits multiple unwanted side effects, including addiction and tolerance. Opioids produce their actions at a cellular level by activating MOR. These receptors are distributed throughout the central nervous system (CNS). The target of this study was the effect of *Smyrnium cordifolium* extract (SCE) on the hippocampus Cornu Ammonis 1 (CA1) aria MOR compared to clonidine.

Materials and methods: Extract of the aerial parts *S.cordifolium* was extracted by Soxhlet method. Addiction was created using the subcutaneous injections of morphine for 7 days. To evaluate the effects of SCE, the mice were divided up 5 groups. The first group (Control) received just morphine. The 2th group received morphine and Clonidine (0.2mg/kg). Groups 3, 4 and 5 were treated morphine and SCE (E1, E2 and E3). In all groups, on the seventh day 30 min after naloxone injection, their brains were perfusion with formaldehyde and removed for immunohistochemical investigation.

Results: The present immunohistochemical of the CA1 hippocampus study showed that group E1, there is a significant difference in MOR optical density compared to the control group at the level ($P<0.05$) and relative to the CLO group at the level ($P<0.001$). However, in groups E2, and E3 the MOR optical density increased compared to the control group and had a significant difference in level ($P<0.001$) and did not have a significant difference compared to the CLO group.

Conclusion: The study showed that with increasing *S.cordifolium* extract concentration, the optical density of MOR in the hippocampus increased, and this increase was dose-dependent. This increase in the CA1 hippocampus MOR optical density may be due to endocytosis or desensitization of MOR in neurons.

Keywords: Hippocampus, *Mu*-opioid receptor, Mice, *Smyrnium cordifolium*

Introduction

Drug addiction in the world has become a socially unpleasant occurrence, causing economic problems, infectious diseases and mental disorders. Therefore, one of the goals of researchers is to find solutions for reducing drug dependence and addiction. Opioids are widely used as one of the best medications to relieve severe acute and chronic pain. But the major problem with long-term opioid use is the issue of

tolerance and dependence. The severe dependence on these drugs physically and mentally makes treatment difficult and makes addiction more difficult (1).

According to certain findings of the mechanisms involved in opioid tolerance and addiction, neurotransmitter systems such as nitric oxide (2), glutamate (3) dopamine (4), and receptors of stimulatory amino acids, especially the receptor (NMDA), are important. The function of glutamate receptors (NMDA) has been proved in the opioid synaptic shaping process (5). Research has exposed that morphine exhibits an analgesic effect by binding to the μ opioid receptor (6). The major opioid receptors in the eukaryotic cell are called Mu (μ), kappa (k), Delta (δ), Sigma (σ) and Epsilon (ε) (7). Some opioid receptors may be subdivided into subcategories, for instance, the Mu opioid receptor has four subtypes (μ1, μ2, μ3, μ4). Modern opioid analgesics generally operate their therapeutic effect through three classic opioid receptors: Mu-opioid receptor (MOR), kappa opioid receptor (KOR), and delta opioid receptor (DOR). These receptors belong to the G-protein coupled receptor (GPCR) superfamily (8). There are three types of endogenous opioid peptides, known as endorphin, dynorphin, and enkephalin which correspond to the aforesaid receptors, respectively. All three peptides are mighty of binding to each of the receptors with different affinities (9). Endorphin predominantly binds to MOR and DOR, enkephalin to DOR, and dynorphin to KOR. Stimulation of opioid receptors by endogenous or exogenous agonist in neurons is associated with the modulation of adenylyl cyclase activity (10), the opening of potassium channels (11), the inhibition of calcium channels (12), and the activation of mitogen-activated protein kinases (MAPKs) (13). Although MOR agonists are exclusively operative as wide-range pain relievers, it has been very difficult to achieve a perfect separation of analgesia from many problematic side effects. The opioid system is critical for inhibitory modulation of pain transmission. Opioid analgesics (for example morphine), which mostly aim Mu-opioid receptors, remain the most powerful analgesics available for pain relief. However, their chronic use may lead to the development of anti-nociceptive tolerance. Blockade of δ-opioid receptors results in enhanced morphine analgesia and reduced tolerance (14), suggesting interaction between DORs and MORs in the pain pathway. The Mu and Kappa opioid receptor are commonly associated with the control of sensation and stimulation (15). Human studies indicated that mu receptor motivation produced dysphoric result and a certain grade of anxiety (16). The evidence in rodents showed that mu opioid receptors activation produced anxiety and depression effects (17). The primary cause of death from opioid overdose is respiratory depression. The mu-opioid receptor is responsible for both the analgesic and respiratory depressant effects caused by morphine (18). Medicinal herbs have been used to treat all periods of human life. In traditional medicine Iran, *S.cordifolium* is used to treat anxiety, pain, insomnia and withdrawal syndrome (19). A number of medicinal properties namely, diuretic, restorative and counteracting renal calculus, have been described from all parts of the plant. Additionally, the fleshy stems of the plant are used as vegetables (20). The genus *Smyrnium* belongs to *Umbelliferae* family is represented in the flora of Iran by only *Smyrnium cordifolium* Boiss species (Avendol), which is a biennial herb and widely distributed in the Zagrose Mountains at an altitude of 1400-2000m in the west and northwest of Iran. This plant has many nutritional and medicinal benefits (21).

There are some reports on the analysis of *S.cordifolium* extract containing curzerenone (19). So that, in a study of *S.cordifolium* of essential oil, identification constituents was made by GC/MS. This oil was characterized by a high content of sesquiterpenes, mostly oxygenated

sesquiterpenes with curzerene (16.9%) and curzerenone(33.8%) as major constituents (20).
Since no studies have been performed on the effects of *S.cordifolium* aerial parts extract on the central nervous system, especially opioid receptors. Therefore, the aim of this study was to investigate the effects of *S.cordifolium* extract on the Mu-opioid receptors of mice compared to clonidine.

Methods and Materials

Extraction method

The aerial parts (flower, leaf, and stem) of *S.cordifolium* used for the present study were collected during the flowering stage between February and March, 2017 from of the Ghalajah Mountains of Ilam province in West of Iran. A voucher sample of the collection was recognized by Dr. Attar F. (Tehran University, Biology, Iran) and was deposited at the department of Biology, Ilam Azad University, Iran. The aerial parts of plant were dried in shade for a week and exposed to air flow at room temperature, then powdered with electric mill. The powder plant extract was prepared by Soxhlet extraction method. Each 15g of powder was crushed into a thimble paper and extracted with 250ml of 70% ethanol. The process of extraction continues for until the solvent in the siphon tube of an extractor become colorless (about 48 hours). In order to evaporate excessive solvent, the sample was then concentrated in a rotary vacuum distiller under negative pressure at 50 °C. The extract was incubated at 30-40 °C until the solvent was completely isolated from the extract and powdered. The powder was preserved in sterile bottles at -20°C during experiments (22).
Experiment was performed on forty adult male mice (25-30g) obtained from the Pasteur Institute (Tehran, Iran). After a week of adaptation to the lab environment, the animals were divided into five groups, each group consisting of eight numbers. The animals were individually housed in eight per cages with a 12/12 hours light/dark cycle and were allowed free access to food and water. Experiments were carried out according to a protocol approved by the Animal Care Committee at Medicine Ilam University and in accordance with policies and guidelines of the Medicine Ilam University Council on Animal Care.
Morphine and Naloxone ampoules and Clonidine hydrochloride tablets were obtained from Tolid Daru Co, Tehran-Iran.

Development of addiction to morphine in mice

The mice were injected subcutaneously with morphine twice daily for 7 days. The dose of morphine on days 1 & 2 were 2.5 mg/kg; this dose was doubled every day after that to reach on day 6 a total dose of 40 mg/kg. On day seven, the mice received the last injection of morphine, 50 mg/kg. Previous studies have shown that this method can addict well.

Effect of co-administration of SCE and morphine

First group Control: this group of mice received just morphine. The second group clonidine hydrochloride (CLO, 0.2 mg/kg i.p.) was administered concurrently with morphine, twice daily for 7 days. The third group (E100), Fourth (E200) and fifth (E300), were treated with different dose's SCE (100, 200, 300mg/kg, Gavage orally) respectively and morphine (23).

Immunohistochemistry (IHC) protocol

Animals were anesthetized with xylene/ketamine (0.2/0.1 ml i.p.), and perfused through the aortic arch with 10 ml 0.9% warm saline, followed by 10 ml 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) in 20-30 minutes. The brain was removed and post-fixed for 2 hours at 4°C in 4% paraformaldehyde in 0.1M PB in 24h. The tissue brain was then transferred to 10% paraformaldehyde in

0.1M PB in 24h. They have been dehydrated by graded ethanol series of 30, 50, 70, 90 and 100%. Tissue brain were clearing with xylene and embedding in warm paraffin. Then a microtome was used to collect serial coronal thickness of 7μm sections through hippocampus. Sections were mounted on gelatine coated slides. All sections were cut 1 day before immunostaining and were attached to the slide by heating in a 60°C oven for 1 hour. All slides were deparaffinised in xylene (three changes of xylene, 2 minutes each), hydrated in graded alcohols (absolute, 95%,and 70% ethanol for 2 minutes each), and placed in Tris buffered saline (TBS; 0.05 mol/L Tris base, 0.15 mol/L NaCl, Triton X-100 4 drops/L, pH 7.6) before antigen recovery and immunostaining. Antigen retrieval was performed with 0.05% trypsin in calcium chloride pH 7.8 at 37´C for 30 minutes. All immunohistochemical staining procedures were performed in dark and humidified chamber to avoid tissue drying out. Then wash in TBS and proceed with staining (24).

The slides were incubated in primary antibody (Anti-Mu Opioid Receptor antibody [UMB3] ab134054; Abcam) 1:200 Tris/EDTA for 24h at 4C. Appropriate Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) ab150077 conjugated secondary antibody raised in Goats was used at 1:100 Tris/EDTA dilutions (Abcam) for 30 minutes at 4C. Wash slides in PBS three times for 5 min. Incubated slides in 1 μg/mL DAPI (DNA stain) for 1 min then rinsed with PBS. The sections were then treated with graded alcohols, and xylene, placed on coverslips with Entellan cytology. Slides were stored in the dark at -4°C until imaged. Specimens were imaged with a fluorescence microscope (CX41, Olympus Germany) and software Image J.

Statistical analysis

SPSS 19 and Excel 2015 were used for statistical analysis. The optical density of MOR was quantified using ImageJ software. Statistical analysis was performed to compare optical density data, using one-way analysis of variance (ANOVA) and the T-test as post hoc analysis for the different groups. The level of significant was set at $P<0.01$.

Results

Figure 1 shows representative immunohistochemical of SCE treated mice used to analyses Mu-opioid receptor (MOR) optical density in this study. This figure is part of the brain of morphine-receiving mice in the control and clonidine groups, which were stained with Mu antibody and then photographed with a fluorescence microscope. Due to the fact that in the CA1 region, the optical density of MOR was observed with greater clarity and number, therefore, this area was selected to examine the density of the MOR.

Figure 2 shows the immunohistochemical results of Mu opioid receptor in the CA1 region of the mice brain affected by the primary Anti-Mu EPR18881 antigen and the secondary antibody Alexa Fluor® 488 in groups E1, E2 and E3, which were analyzed by Image J software.

Also, statistical analysis of figure 3 shows that means in group E1 (receiver of S.cordifolium extract with a concentration of 100 mg/kg, images G, H, I), there is a significant difference in optical density compared to the control group at the level ($P<0.05$) and relative to the CLO group (the simultaneous recipient of morphine and clonidine with a concentration of 0.2mg/kg in images D, E, F) at the level ($P<0.001$). However, in groups E2 (receiver of S.cordifolium extract with a concentration of 200 mg/kg, images J, K, L), and E3 (receiver of S.cordifolium extract with a concentration of 300 mg/kg, images M, N, O), the optical density increased compared to the control group and had a significant difference in level ($P<0.01$ and $P<0.001$, respectively) and did not have a significant difference compared to the CLO group.

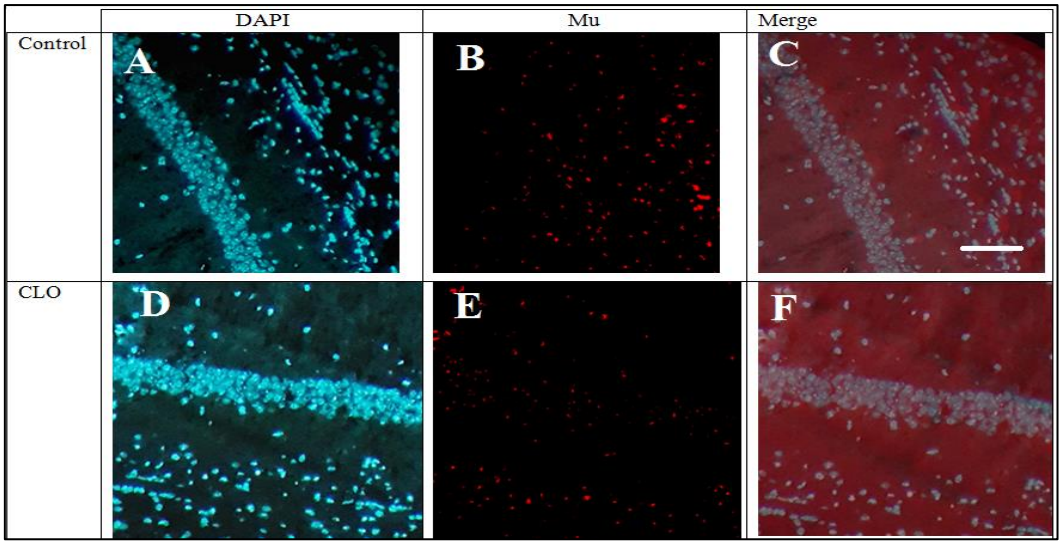

Figure 1. Immunohistochemistry of the mouse brain hippocampus in the control and clonidine groups: The recipient of Mu has been under the primary influence of Anti-Mu EPR18881 and secondary antibody Alexa Fluor® 488. Magnification of 200X fluorescence microscope, Scale: 75µm, n = 8 per group
A: Image of the CA1 area of the hippocampus in the control group, painted with DAPI and antibody Mu, and photographed with a blue filter to view the nuclei. B: Picture of the CA1 hippocampal area of the control group, painted with Mu antibodies and filmed with a red filter to see Mu receivers. C: Image of CA1 area of merged hippocampus A, B with ImageJ software in the control group. D: Image of CA1 hippocampal region in clonidine group stained with DAPI and antibody Mu and photographed with blue filter to view nuclei. E: CA1 hippocampal group CA1 image stained with Mu antibody and filtered with red filter to track Mu receptors. F: Image of CA1 area of merged hippocampus D, E with ImageJ software in clonidine group

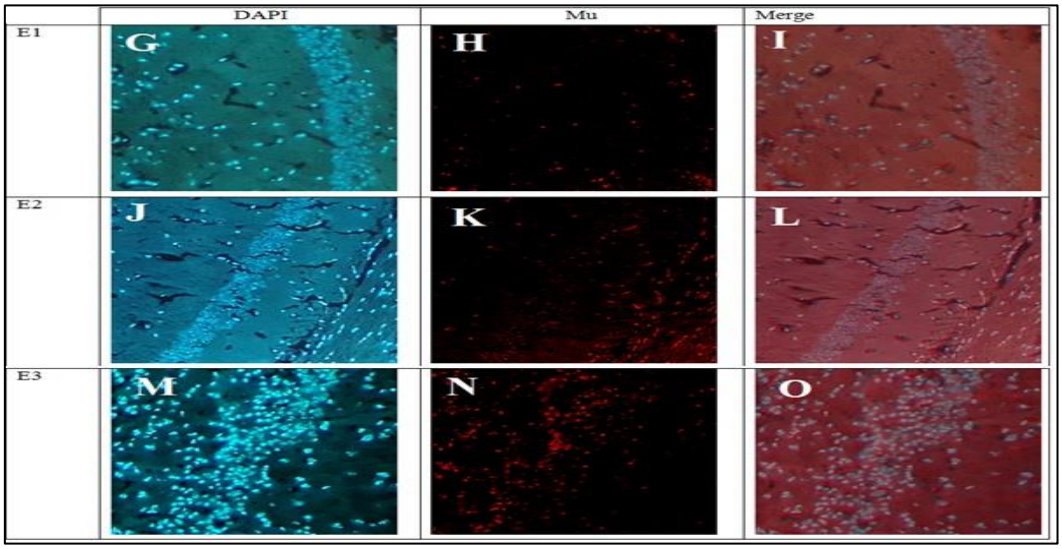

Figure 2. Immunohistochemistry of the mouse brain hippocampus in E1, E2 and E3 groups: The Mu opioid receptor is affected by primary antibody Mu EPR18881 and secondary antibody Alexa Fluor® 488. Magnification of 200X fluorescence microscope, Scale bar: 75µm, n = 8 per group. G: Picture of the CA1 area of the hippocampus in E1 group stained with DAPI and Mu antibody than photographed with a blue filter to view the nuclei. H: Image of CA1 hippocampal region E1 group stained with Mu antibody and filmed with red filter to track Mu receptors. I: Image of CA1 area of merged H, G hippocampus with ImageJ software in E1 group.
J: Picture of the CA1 area of the hippocampus in the E2 group stained with DAPI and Mu antibody, then photographed with a blue filter to view the nuclei. K: Image of CA1 hippocampal area E2 group stained with Mu antibodies and photographed with red filter to track Mu receptors. L: Image of CA1 area of merged hippocampus K, J with ImageJ software in E2 group. M: Image of the CA1 area of the hippocampus in the E3 group, stained with DAPI and Mu antibody, then photographed with a blue filter to view the nuclei. N: Image of CA1 hippocampal area E3 group stained with Mu antibody and filmed with red filter to track Mu receptors. O: Image of CA1 area of merged M, N hippocampus with ImageJ software in group E3.

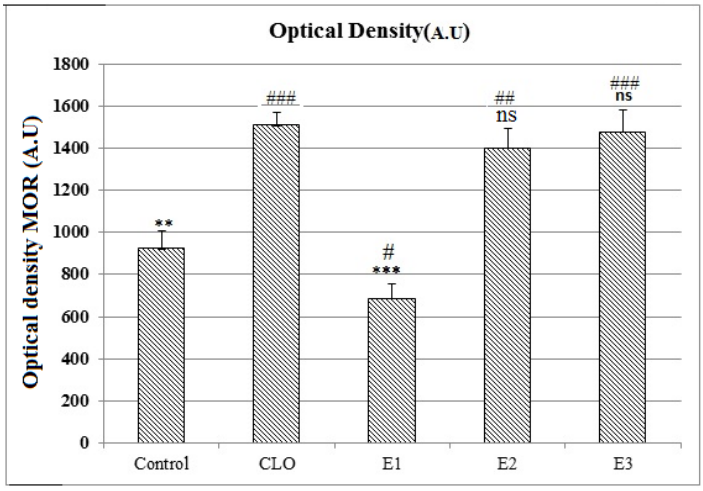

Figure 3. The graph shows the means of optical density of the Mu receptors in groups control, CLO(Clonidine) and E1(receptor of SCE with a concentration 100 mg/kg), E2(receptor of SCE with a concentration 200 mg/kg), E3(receptor of SCE with a concentration 300 mg/kg). #P< 0.05). ##P< 0.01, ###P< 0.001, **P< 0.01, ***P< 0.001. ns: There are no significant differences. Results are means ± SEM (n= 8).

Discussion

The results of the present study clearly demonstrate that repeated administration with *S.cordifolium* extract increased Mu opioid receptor optical density in most brain regions, including the hippocampus. The magnitude of the effect depended upon of extract concentration. We have previously reported that the administration of *S.cordifolium* extract at different concentrations in morphine-dependent mice reduced the symptoms of addiction withdrawal syndrome, such as jumping, rearing, and teeth chattering (19), as well as motor activity, tolerance and dependence (25). Dependence and tolerance can be caused by a decrease in the number of opioid receptors, or a decrease in their reactivation, and a change in the stimulus response that our results are consistent with these studies.

The most effective opioid pain relievers are agonists of the Mu opioid receptors (MOR). Agonists of the MOR, such as morphine are widely expended for the treatment of moderate to severe pain because of their high efficacy. Nevertheless, the dose required to achieve adequate pain relief often elicits multiple unwanted side effects, including withdrawal syndrome, and tolerance. The withdrawal syndrome and tolerance effect of opioids are especially concerning, because it is the origin reason of addiction by opioid overdose. The identification and improvement of safer analgesic agents may play a main role in combatting the opioid epidemic. Opioid receptors such as Mu and Kappa are known to control neurotransmission of various peptidergic neurons, but changes in number and density and their potential role in regulation of neuronal cell communications is unknown (24).

According to immunohistochemical images and their analysis by Image J software based on the present study showed an increase in the density of hair receptors relative to the control group. Therefore, it can be concluded that with the increase of opioid receptors, especially hair receptors, the rate of tolerance and dependence decreases.

In 2001, Harris and colleagues found that chronic opioid use caused desensitization of pre-synapse Mu receptors and reduced the inhibitory effects of opioids on these nerves (26). Also, in our study, it is possible that the optical density of opioid receptors in some groups is increased due to their desensitization by the extract.

Chronic morphine treatment induces Mu opioid receptors abundance in different brain regions (27). Based on the results of this study, the optical density of the Mu opioid receptors in the CA1 region of

hippocampus was lower in the control group than in the clonidine group. Thus, it can be concluded that *S.cordifolium* extract may have increased the optical density of Mu opioid receptors compared to the control group. These results are consistent with Harris et al., 2001 and Fujita et al., 2015 results. Furthermore, in 2010, Gupta showed in a study that opioids increase Mu opioid receptors (28), which is consistent with our findings.

In 2012 Garzon et al., showed that the CA1 area of the hippocampus is affected by some drugs that affect pain and anxiety (28). Our study also found that the Mu opioid receptor optical density in the CA1 region of the control group was lower than that of the clonidine group. Therefore, it can be concluded that *S.cordifolium* extract may have increased the optical density of Mu receptors compared to the control group.

Endocytosis of G-protein receptors is a cellular event in which the receptor is generally activated by agonist compounds. Basically, this process involves the formation and movement of vesicles originating from the plasma membrane into the cytoplasm. The activated receptors then release the cell surface into the vesicles and travel through various pathways that lead to their destruction or recycling into the plasma membrane. In fact, endocytosis is a mechanism for the cessation of various intracellular signaling cascades that begin with activation of the receptor (30). The present study, based on the Campa study in 2015, changed the optical density of Mu opioid receptors, which may have been due to endocytosis or desensitization of Mu receptors.

Conclusion

In general, the present immunohistochemical study indicated that with increasing *S.cordifolium* extract concentration, the optical density of Mu opioid receptors in the hippocampus increased, and this increase was dose-dependent. *S.cordifolium* extracts such as clonidine relieve pain caused by addiction withdrawal syndrome, and these effects are likely to be related to increased receptor light density in the CA1 hippocampus area of the brain.

Acknowledgements

The authors would like to thanks Dr. Moayeri, Director of the Department of Histology at Ilam University of Medical Sciences. We also thank Dr. Azizi (Ilam University of Medical Sciences) for valuable revision advice and proofreading.

Conflict of Interest

The authors declare no conflicts of interest.

References

1. Abbasi Maleki S. Effect of ethanolic extract of Safflower on naloxone-induced morphine withdrawal signs in mice. Adv Herb Med. 2015; 1(4):9-15. doi: 10.22038/IJBMS.2017.9277.
2. Ozdemir E, Bagcivan I, Durmus N, Altun A, Gursoy S. The nitric oxidecGMP signaling pathway plays a significant role in tolerance to the analgesic effect of morphine. Can J Physiol Pharmacol. 2011; 89(2):89-95. doi.org/10.1139/Y10-109.
3. Capone F, Adriani W, Shumilina M, Izykenova G, Granstrem O, Dambinova S. Autoantibodies against opioid or glutamate receptors are associated with changes in morphine reward and physical dependence in mice. Psychopharmacology. 2008;197 (4):535-48. doi: 10.1007/s00213-007.
4. Zarrindast, MR, Bahreini T, Adl M. Effect of imipramine on the expression and acquisition of morphine-induced conditioned place preference in mice. Pharmacol Biochem Behav. 2002;

73(4):941-9. doi: 10.1016/s0091-3057(02)00951-6.
5. Noda Y, Nabeshima T. Opiate physical dependence and N-methyl-Daspartate receptors. Eur J Pharmacol. 2004; 500(1-3):121-8. doi: 10.1016/j.ejphar.2004.07.017.
6. Kest B, Palmese CA, Hopkines E, Adler M, Juni A. Assessment of acute and chronic morphine dependence in male and female mice. Pharm Biochem Behav. 2001; 70: 149-56. doi: 10.1016/s0091-3057(01)00600-1.
7. Kieffer BL, Evans CJ. (2009) Opioid receptors: from binding sites to visible molecules in vivo. Neuropharmacology. 2009; Suppl 1:205-12. doi: 10.1016/j.neuropharm.2008.07.033.
8. Spetea M. Opioid receptors and their ligands in the musculoskeletal system and relevance for pain control. Curr Pharm Des.2013; 19(42):7382-90. doi: 10.2174/13816128113199990363.
9. Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM. Endogenous opioids: biology and function. Annu Rev Neurosci. 1984; 7:223-55. doi: 10.1146/annurev.ne.07.030184.001255.
10. Childers SR. Opioid receptor-coupled second messenger systems. Opioids. 189-216. doi: 10.1016/0024-3205(91)90154-4.
11. Henry DJ, Grandy DK, Lester HA, Davidson N, Chavkin C. Kappa-opioid receptors couple to inwardly rectifying potassium channels when coexpressed by Xenopus oocytes. Mol Pharmacol. 1995; 47 (3):551-7. PMID: 7700253.
12. Macdonald RL, Werz MA. Dynorphin A decreases voltage-dependent calcium conductance of mouse dorsal root ganglion neurones. J Physiol (Lond). 1986; 377:237-49. doi: 10.1113/jphysiol.1986. sp016184.
13. Bruchas MR, Xu M, Chavkin C. Repeated swim stress induces kappa opioid-mediated activation of extracellular signal-regulated kinase 1/2. Neuroreport. 2008; 19(14):1417–1422. doi: 10.1097/WNR. 0b013e32830dd655
14. Schiller PW. Bi- or multifunctional opioid peptide drugs. Life Sci. 2010; 86:598–603. doi:10.1016/j.lfs.2009.02.025.
15. Carroll FI1, Carlezon WA Jr. Development of kappa opioid receptor antagonists. J Med Chem. 2013; 28;56(6):2178-95. doi: 10.1021/jm301783x.
16. Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E, Henderson G. Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. Mol Pharmacol. 2006; 70(2):676-85. doi: 10.1124/mol.106.022376.
17. Lipiński, PFJ, Jarończyk, M, Dobrowolski JC. Molecular dynamics of fentanyl bound to μ-opioid receptor. J Mol Model. 2019; 25, 144. doi.org/10.1007/s00894-019-3999-2.
18. Dahan A, Sarton E, Teppema L, Olievier C, Nieuwenhuijs D, Matthes HW, Kieffer BL. Anesthetic potency and influence of morphine and sevoflurane on respiration in mu-opioid receptor knockout mice. Anesthesiology. 2001; 94:824-32. doi: 10.1097/00000542-200105000-00021.
19. Nazari P, Yaghmaei P, Rangin A, Abbasi, N. Effects of curzerene and Smyrnium cordifolium Boiss extract on addiction withdrawal syndrome in mice. J Herbmed Pharmacol. 2018; 7(4)280-6. doi: 10.15171/jhp.2018.42.
20. Amiri H, Khavarinejad RA, Rustaiyan A. [Chemical composition of essential oil and study of secretory anatomy from Smyrnium cordifolium boiss]. Pajouhesh Sazandegi J. 2007; 20: 11-6. (Persian).
21. Zargari, A. [Iranian medicinal plants]. Tehran University Publications, Tehran, 1997; 1: 558-65. (Persian).

22. Feily, A. & Abbasi N. The inhibitory effect of *Hypericum perforatum* extract on morphine withdrawal syndrome in rat and comparison with clonidine. Phytotherapy Research Phytother. Res. 2009; 23: 1549-52. doi: 10.1002/ptr.2807.
23. Abbasi N, Mohammadpour S, Karimi E, Aidy A, Karimi P, Azizi M, Asadollahi K. Protective effects of *Smyrnium cordifolium* boiss essential oil on pentylenetetrazol-induced seizures in mice: involvement of benzodiazepine and opioid antagonists. J Biol Regulator Homeostat Agent. 2017; 31 (3): 683-9.
24. Ehrlich AT, Semache M, Gross F, Da Fonte DF, Runtz L, Colley C, Kieffer B L. Biased signaling of the mu opioid receptor revealed in native neurons. iScience 2019;14: 47-57. doi: 10.1016/j.isci. 2019.03.011.
25. Yaghmaei et al. The role of *Smyrnium cordifolium* Boiss extract and curzerene on withdrawal syndrome in mice. Cell Mol Biol. 2019; 65(7): 77-83. doi.org/10.14715/ cmb/2019.65.7.14.
26. Harris RE, Clauw DJ, Scott DJ, McLean SA, Gracely RH, Zubieta JK. Decreased central mu-opioid receptor availability in fibromyalgia. J Neurosci. 2007; 27:10000-6. doi:10.1523/ jneurosci.2849-07.2007.
27. Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E, et al. Increased abundance of opioid receptor heteromers after chronic morphine administration. Sci Signal. 2010; 3(131):ra54. doi: 10.1126/scisignal.2000807.
28. Fujita W, Gomes I, Devi LA. Heteromers of m-d opioid receptors: new pharmacology and novel therapeutic possibilities. Br J Pharmacol. 2015; 172:375-87. doi:10.1111/ bph.12663.
29. Rodríguez-Muñoz M, Sanchez-Blazquez P, Vicente-Sanchez A, Berrocoso E, Garzon J. The mu-opioid receptor and the NMDA receptor associate in PAG neurons: implications in pain control. Neuropsychopharmacology. 2012;37: 338-49. doi: 10.1038/npp.2011.155.
30. Campa VM, Capilla A, Varela MJ, dela Rocha AMA, Fernandez-Troyano J C, Barreiro RB, et al. Endocytosis as a biological response in receptor pharmacology: Evaluation by fluorescence microscopy. PLoS ONE 10(4): e0122604. doi: 10.1371/journal.pone.0122604.