ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Study of the Analgesic Effect of Hydro-alcoholic Extract of Red Lentil in Rat by Formalin Test.

Ardeshir Arzi[1], Neda Sistani Karampour[2], Zahra Nazari Khorasgani[2], and Amir Dashtizadeh[3].

[1]Department of Pharmacology.Toxicology, School of Pharmacy Physiology Research CenterJundishapur University ofMedical Sciences, Ahvaz, Iran.
[2]Department of Pharmacology.Toxicology, School of Pharmacy ,Jundishapur University of Medical Sciences, Ahvaz, Iran.
[3]School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

ABSTRACT

Due to the increased role of medicinal plants in therapy, the aim of this research was to study the analgesic effect of hydroalcoholic extract of the red lentil and to compare it with morphine and aspirin as common analgesics. In this experimental study, Wistar male rats were divided into six groups of 6 rats, randomly. Negative control group (normal saline 5ml/kg); two positive control groups morphine (2.5 mg/kg) and aspirin(300mg/kg) and three treated groups (100, 200 and 400 mg/kg) received a single dose of hydroalcoholic extract of red lentil via intrapritoneal respectivals. In this study, the analgesic effect was investigated by using formalin test. The results revealed that the red lentil extract has dose-depended analgesic effect was 200 mg/kg. There was not any significant difference between 200mg/kg and 400mg/kg of extract in all of time of study. Our results showed that the analgesic effect of best effective dose (200mg/kg) on acute pain had not significant difference with morphine and aspirin in other hand the effect of extract (200mg/kg) on chronic pain was less than morphine and more than aspirin. The results indicate that the analgesic effect of hydroalcoholic extract of red lentil can probably be due to the influence of the antioxidants of this extract.

Keywords: Red lentil extract,asprim,morphin, Pain, Formalin test, Rat.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Herbal medicine has long been common in ancient civilizations and it is practicing today in different forms, including the use of herbal preparations or their total extracts. Given the expanding demand for herbal medicine all over the world and a rich source of medicinal herbs in Iran, research in this area seems necessary [1-3].

The lentil (*Lens culinaris*) is an herbaceous annual plant of the legume family with short and partially branched stalk in bright green. It is 15 and 75 cm in height but in normal growth conditions, most of the genotypes of the plant are 25 and 40 cm [4,5].

The plant is grown in most parts of the world. Lentil's chemical compounds include flavonoids, tannins, catechin, and epicatechin. Lentil also contains important minerals, vitamins (B) and protein . Since several studies have shown that legumes such as red lentil contain abundant polyphenols, it is expected that this audible pulse may have an appropriate analgesic effect [6,7].

In the present study, the effect of ethanolic extract of red lentil on pain was assessed using the formalin test. If proofed, this research can be an introduction to the subsequent studies on animal models and ultimately on human model according to the specific terms and conditions for future use as an analgesic.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 120-150 grams were obtained from the Laboratory Animals Center of Jundishapur University of Medical Sciences. Animals were kept inside standard cages in the animal room at a temperature of 23±2 °C and humidity of 40%-50%, with 12 hours light/dark cycle. During the study, the rats had access to animals' compressed food supplied by Pars Tehran animal food company and purified tap water. The animals were randomly divided into 7 groups of 9 rats and each animal was used only once during the study.

Preparation of Extract

The maceration method was used in this study for extraction of red lentil seed. The seeds were pulverized by an electric grinder and 200 grams of the plant powder was poured in a beaker. Then, 70% ethanol was added to the powder to cover its surface up to 2 cm. Finally, the container's aperture was covered with aluminum foil and stored for 72 hours. During soaking, the extract containing container was mixed well three times a day and the soaking product was collected in a container after 72 hours following isolation of the pulp. The pulp was then washed again with 70% ethanol and added to the previous extract. The obtained extracts were purified firstly by cotton and then by Whatman filter paper and concentrated by vacuum distillation apparatus. The resulting concentrate was put in an oven at 30-40 °C and become a dried extract after complete loss of the solvent. The dried product weighed and maintained in a dark glass container in a cool and dry place [8].

Formalin test

The method of Dubuisson and Dennis was used to perform the test. First, each animal was put in a 22×22×22 cm Plexiglas-made chamber on a formalin test device. There was a mirror at 45 degrees angle under the glassy surface of the device, making easy the observation and evaluation of the animals' feet position [9].

The animals were divided into 7 groups of 9 each and were undergone intraperitoneal administration as follows: a negative control group with normal saline (5 mL/kg), the positive control groups with aspirin and morphine (300 mg/kg and 2.5 mg/kg), and the experimental groups receiving the extract at doses 100, 200, and 400 mg/kg. After half an hour, 50 mL formalin 2.5% was injected subcutaneously at the bottom of the animals' paw and each animal was immediately studied in terms of the pain severity. It should be noted that the time 0 to 5 min after formalin injection was considered as the first phase of pain (acute pain) and was accounted as a block. Each rat was evaluated once every 15 seconds and received a score. The pain in the

ISSN: 0975-8585

acute phase was measured 20 times in each animal, and finally the average score of this test was identified for each group. At the same time, the time 15 to 60 min after formalin injection was considered as the second phase of pain (chronic pain); these 45 minutes was divided into 9 blocks of 5 minutes. Therefore, to study the chronic phase, each rat was examined 20 times in each 5-minute block and totally each rat was evaluated 180 times for 9 blocks in terms of pain severity; then the mean score was calculated for each block. The pain was scored as follows:

a) If the animal walked and sat regardless of the injected foot and the foot well tolerated the animal weight, it had no pain and scored zero.
b) If the animal did not easily put the injected foot on the glass surface and tried to put its weight on the uninjected foot, it had pain and scored 1.
c) If the animal raised the injected foot from the surface and tried to not put it on the surface and bear its weight completely on the other foot, it scored 2.
d) If the animal licked, bit, and severely shook the injected foot, it scored 3.

Statistical Methods

The data were expressed as mean ± SEM. The Kruskal-Wallis nonparametric test was used to compare the means. Homogeneity of the data was analyzed by the nonparametric K-S sample to determine the distribution type. Since the distribution was normal, the ANOVA parametric test and then the Tukey's test were used to determine the significance of differences between the groups. It should be noted that the General Linear Model was also used and similar results were obtained. $P<0.05$ was considered as the level of significance between the differences [10].

RESULTS

Response to pain in the first phase of the formalin test was significantly reduced by the doses 200 and 400 mg/kg red lentil extract in comparison with the negative control group ($p<0.05$) and no significant difference was observed between the effects of these doses. In the second phase of pain, the doses 200 and 400 mg/kg significantly reduced the response to pain. No significant difference was observed between the dose of 100 mg/kg and the normal saline receiving group (in both phases) (Figure 1).

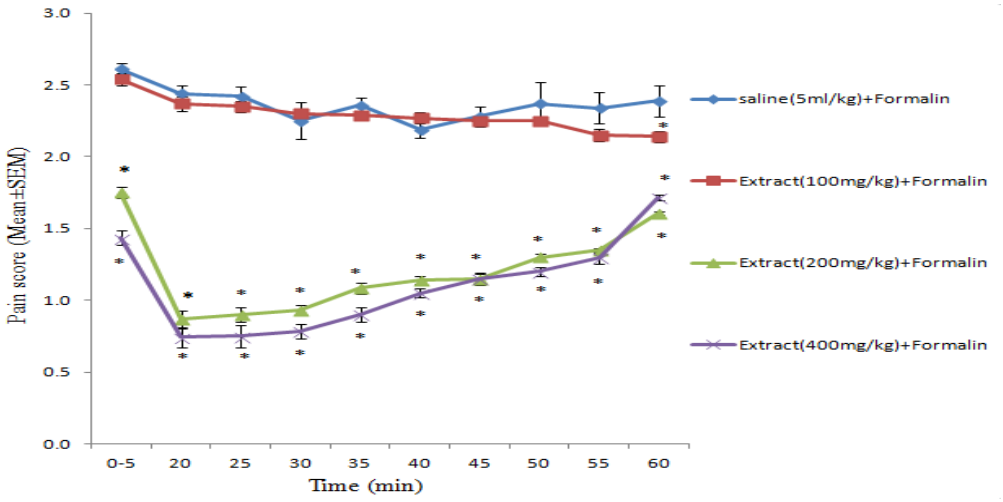

Figure 1: Comparison of the analgesic effects of different doses of hydro-alcoholic extract of red lentil with normal saline

Comparison of analgesic effect (first and second phases of pain) of red lentil hydro-alcoholic extract injected intraperitoneally at different doses (100, 200, 400 mg/kg) and the normal saline receiving group (5 ml/kg), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the normal saline receiving group is significant ($p<0.05$).

ISSN: 0975-8585

In comparison with the dose 100 mg/kg, the doses 200 and 400 mg/kg red lentil extract significantly reduced pain responses in both the first and second phases of pain in the formalin test (p<0.05) and this effect was dose-dependent (Figure 2).

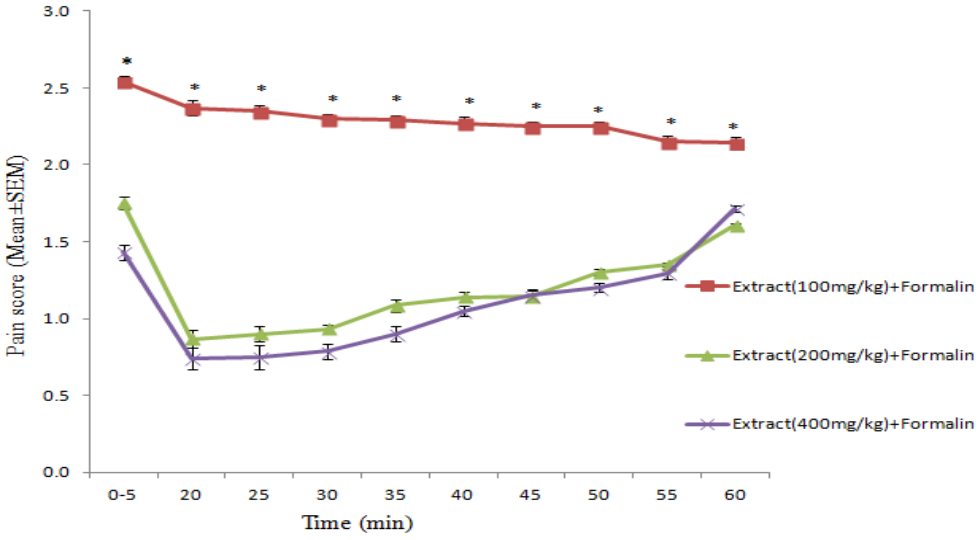

Figure 2: Comparison of the analgesic effects of different doses of hydro-alcoholic extract of red lentil with the most effective dose of the extract

Comparison of analgesic effect (first and second phases of pain) of red lentil hydro-alcoholic extract injected intraperitoneally at different doses (100, 200, 400 mg/kg) and the most effective dose of the extract (200 mg/kg), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the group receiving the most effective dose of the extract is significant (p<0.05).

No significant difference was observed between the effects of these two doses and hence 200 mg/kg was selected as the best dose (Figure 3).

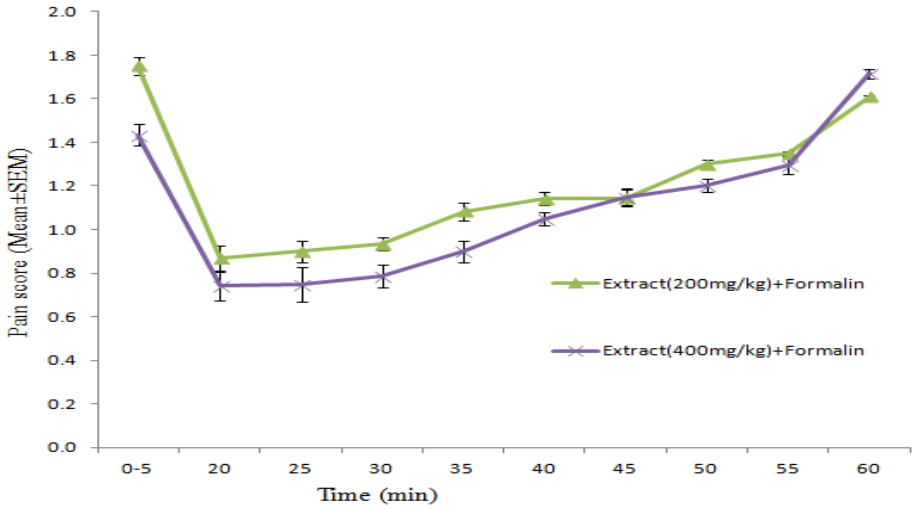

Figure 3: Comparison of the analgesic effects of the doses 200 mg/kg and 400 mg/kg of hydro-alcoholic extract of red lentil

Comparison of analgesic effect (first and second phases of pain) of 200 mg/kg and 400 mg/kg red lentil hydro-alcoholic extract injected intraperitoneally, using the formalin test. Results were expressed as mean ± SEM (n=6).

In comparison with the negative control group, doses 200 and 400 mg/kg of hydro-alcoholic extract of red lentil and the groups receiving morphine and aspirin significantly reduced the pain response in the first

ISSN: 0975-8585

phase of the formalin test (p<0.05), while the dose 100 mg/kg had no effect on the first phase of pain (Figure 4).

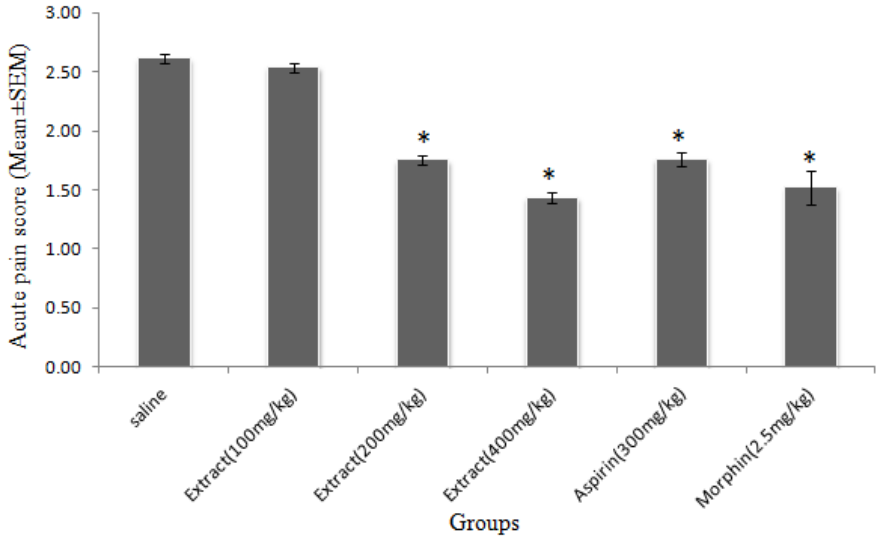

Figure 4: Comparison of the analgesic effects of different doses of hydro-alcoholic extract of red lentil with the positive and negative control groups at the first phase of pain

Comparison of analgesic effect of red lentil hydro-alcoholic extract injected intraperitoneally at different doses (100, 200, 400 mg/kg) and the positive controls (morphine [2.5 mg/kg] and aspirin [300 mg/kg]) and negative controls (5 mL/kg) at the first phase of pain (0 to 5 min), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the normal saline receiving group is significant (p<0.05).

In comparison with the negative control group, doses 200 and 400 mg/kg of hydro-alcoholic extract of red lentil and the morphine and aspirin receiving groups significantly reduced the pain response in the second phase of the formalin test (p<0.05) (Figure 5).

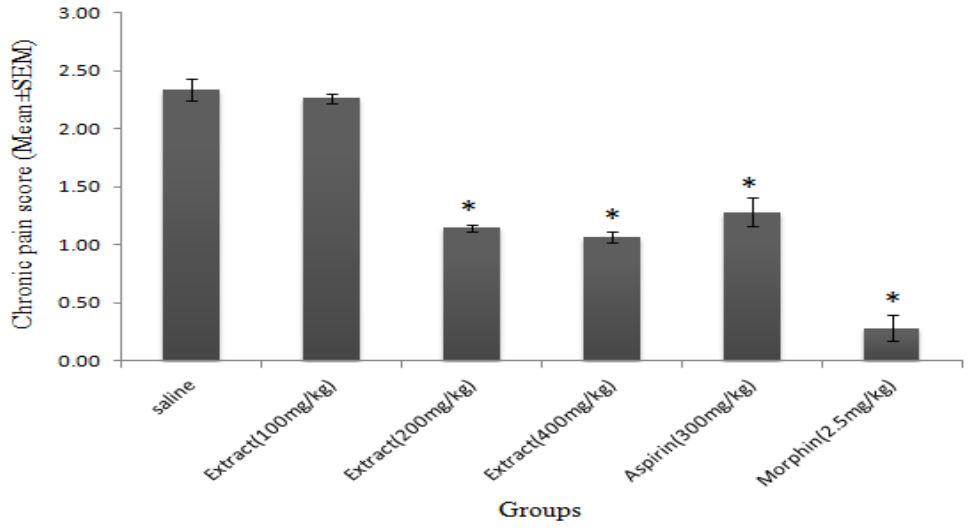

Figure 5: Comparison of the analgesic effects of different doses of hydro-alcoholic extract of red lentil with the positive and negative control groups at the second phase of pain

Comparison of analgesic effect of red lentil hydro-alcoholic extract injected intraperitoneally at different doses (100, 200, 400 mg/kg) and the positive controls (morphine [2.5 mg/kg] and aspirin [300 mg/kg]) and negative controls (5 mL/kg) at the second phase of pain (15 to 60 min), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the normal saline receiving group is significant (p<0.05).

ISSN: 0975-8585

Morphine (2.5 mg/kg) and aspirin (300 mg/kg) significantly reduced pain in both phases of the formalin test as compared to the negative control (p<0.05) (Figure 6).

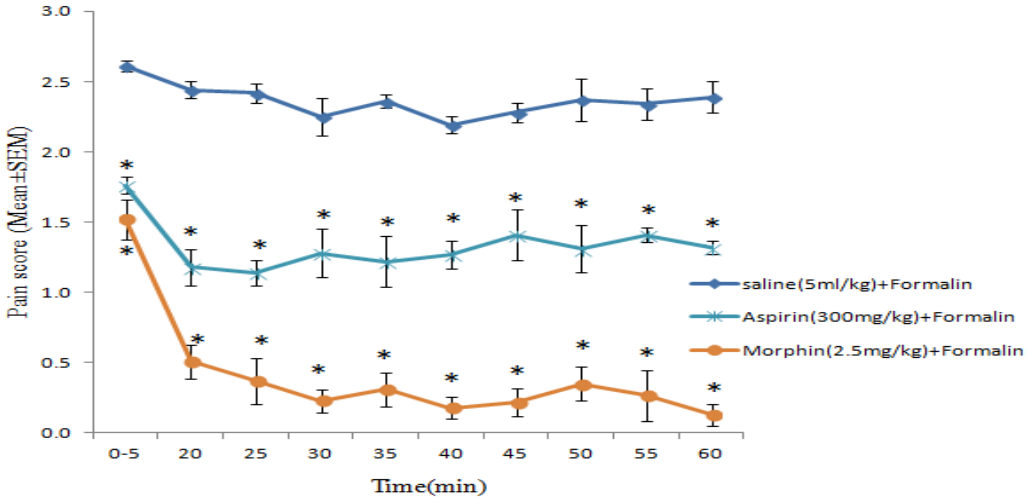

Figure 6: Comparison of the analgesic effect of morphine and aspirin with the normal saline receiving group

Comparison of analgesic effect (first and second phases of pain) of intraperitoneally injected morphine (2.5 mg/kg) and aspirin (300 mg/kg) with the normal saline receiving group (5 mL/kg), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the normal saline receiving group is significant (p<0.05).

Comparison of analgesic dose of 200 mg/kg hydro-alcoholic extract of red lentil with morphine and aspirin showed that the analgesic effect of the extract in the first phase of pain was less than morphine and almost equal to aspirin. No significant difference was observed between the analgesic effect of the extract (200 mg/kg) receiving group and the aspirin and morphine receiving groups in the first phase of pain. In comparison with aspirin, 200 mg/kg extract produced a significant increase in analgesic effect at minutes 15-20 and 25-20 of the chronic phase. There was a significant difference between the analgesic effect of 200 mg/kg extract and morphine during the entire minutes of the chronic phase (Figure 7).

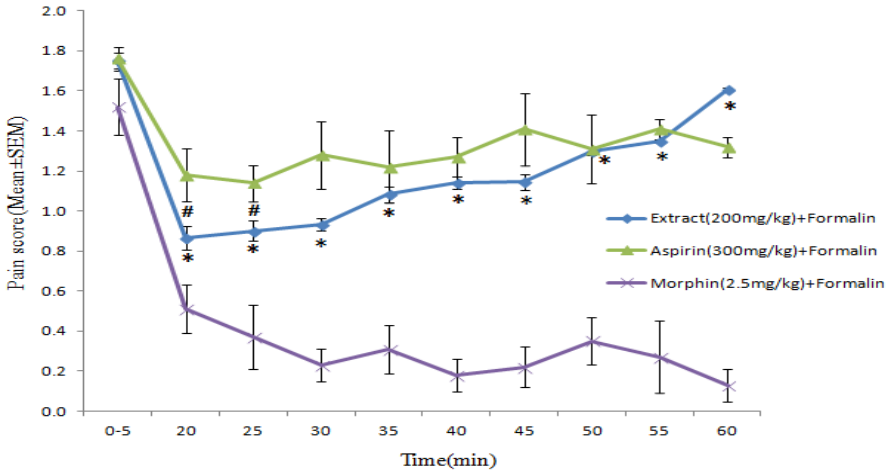

Figure 7: Comparison of the analgesic effects of different doses of hydro-alcoholic extract of red lentil with the positive controls of morphine and aspirin

Comparison of analgesic effect (first and second phases of pain) of intraperitoneally injected red lentil hydro-alcoholic extract at different doses (100, 200, 400 mg/kg) with the positive control groups (morphine [2.5 mg/kg] and aspirin [300 mg/kg]), using the formalin test. Results were expressed as mean ± SEM (n=6). * Difference with the saline receiving group is significant (p<0.05).

ISSN: 0975-8585

DISCUSSION AND CONCLUSION

Pain is a common problem that affects people and a great effort has been made for years to find a way to get rid of it [11].

Medicinal plants have been used so far in the treatment of many human diseases and since synthetic drugs have more harmful effects than herbal medicines, many studies nowadays have focused on therapeutic effects of various plants throughout the world [12]. In this study, the effect of ethanolic extract of red lentil on pain was assessed using the formalin test.

Formalin test is a standard assay to measure the response against a painful stimulus. Subcutaneous injection of formalin results in a biphasic pain which is an important feature of this method for assessing pain. Probably neurotransmitters such as substance P, bradykinin, glutamate, and serotonin are involved in formalin-induced pain. The early phase which begins immediately after formalin injection is the result of direct stimulation of type c sensory fibers. The second phase of pain or the late phase which starts approximately 15 minutes after the injection of formalin is the result of an inflammatory process [13]. This test is being performed to investigate the mechanism of pain and analgesic compounds. Analgesics that exert their effects through the CNS are able to inhibit both phases of the formalin-induced pain; while peripherally-acting analgesics can inhibit only the second phase of pain in the formalin test [14].

Studies of other researchers show that the extract of red lentil contains flavonoids [15,16] which *per. se* have anti-inflammatory and analgesic effects. Their direct effect on the synthesis of prostaglandins has been clearly identified [17]. Flavonoids are inhibitors of nitric oxide synthase and hence prevent NO production which increases following formalin injection [18]. Since NO may be a painful mediator [19], its decrement can lead to an analgesic activity. Studies have also shown that flavonoids are involved in pain modulation via opioid and adrenergic systems [20,21]. Other studies have indicated that flavonoids show their analgesic effects through inhibiting *N*-methyl-D-aspartate (NMDA) receptors, resulting in a decrease in intracellular calcium followed by a reduction in nitric oxide synthase and calcium-dependent phospholipase A2 activities and hence decreased NO and prostaglandins. Inhibition of phospholipase A2 activity blocks the conversion of phosphatidic acid to arachidonic acid and hence synthesis of prostaglandins [22]. According to the existing evidence, in response to inflammatory stimuli, flavonoids inhibit the production of prostaglandins from arachidonic acid through inhibition of cyclooxygenase [23].

Given the high phenolic content of red lentil extract and analgesic and anti-inflammatory effects of polyphenolic compounds, and since flavonoids reduce neurogenic pain and inflammation through activation of several nerve pathways; it is likely that the mechanism of this plant in producing analgesic effect performs through phenolic compounds; to achieve the exact mechanism requires further studies.

Different results obtained from various studies on the analgesic effect of a compound can arise from differences in methodology and in measurement of the pain severity.

Many factors influence the results of studies on analgesics; among them the following can be mentioned:

- Type and species of animals used
- Time of day that the test is conducted
- Type of pain stimulus
- Area of the body to create pain
- Intervals between pain stimuli
- The initial temperature of stimulated area in the thermal stimulation assay. For example, early temperature of tail skin in the Tail Flick Test can alter the test results.

The results of a study indicated that 2.5-10 mg/kg morphine inhibits the pain in the both phases I and II in a dose-dependent manner, while 300-400 mg/kg aspirin inhibits the pain in the second phase. In the study by Hanskar *et al*. on the analgesic effects of NSAIDs in a group of white small mice using the formalin test, aspirin inhibited both phases of the pain [24].

These results are consistent with the present study. This difference can probably be attributed to the dual function of aspirin; so that it reduces pain centrally through inhibition of CNS prostaglandins, increasing serotonin, and decreasing $5HT_2$ receptors, as well as peripherally through direct action on nociceptors in the inflamed area [25,26]; to achieve the exact mechanism requires further investigations.

In the present study, extracts of red lentils at doses 100, 200, and 400 mg/kg were intraperitoneally injected to the rats half an hour before formalin injection and their analgesic effects were compared with those of negative controls (saline, 5 mL/kg) and positive controls (morphine [2.5 mg/kg] and aspirin [300 mg/kg]). According to the results, the doses 200 mg/kg and 400 mg/kg had the maximum analgesic effect, however, since no significant difference was observed between them and due to lower risk of side effects of 200 mg/kg than 400 mg/kg, the former was considered as the optimal dose.

As a central-acting analgesic, morphine can inhibit both the first and second phases in formalin-induced pain. Given the anti-oxidative effect of this plant, and its high phenolic content, as well as anti-inflammatory effects of polyphenolic compounds found in plants and the relationship of anti-oxidative effects with anti-inflammatory and analgesic effects, it seems that the analgesic mechanism of this plant is exerted similarly; to achieve the exact mechanism requires further investigations.

Accordingly, it appears that the plant can be used as an adjuvant for analgesic effect of NSAIDs and morphine and hence can reduce their consumption and ultimately may prevent or decrease their side effects and toxicity.

REFRENCES

[1] Harrison. T.R., et al. principal of internal medicine, 12th Ed, Mc Graw-Hill, p.93-95, 1991
[2] Melzack R. Can J Expp Sychol 1993; 47(4): 615-616.
[3] Hoffert marvin J. Neurologic Clinics 1989; 7(2): 183-185
[4] Mozaffarian V. A dictionary of Iranian plant names. Tehran: Farhang Moaser; 1995. p. 443-444.
[5] Takruri HR, Issa AY. Mediterranean J Nutr Metabol 2013;6(1):3-16.
[6] Holst B, Williamson G. Curr Opin Biotechnol 2008; 19(2):73-82.
[7] Takruri HR, Issa AY. Mediterranean J Nutr Metabol 2013;6(1):3-16.
[8] Amarowicz R, Estrella I, Hernández T, Dueñas M, Troszyńska A, Kosińska A, et al. Int J Mol Sci 2009;10(12):5513-27.
[9] Roslan JH, Tjalsen A, Machle B, et al. Pain 1990; 42(2): 235-242.
[10] Dubuisson D,Dennis SG. Pain 1997; 4: 161-174.
[11] Hunskaar S, Fasmer OB, Holex. Neurosci Methods 1985; 4(1): 69-76.
[12] Zou Y, Chang SK, Gu Y, Qian SY. J Agr Food Chem 2011;59(6):2268-76.
[13] Amarowicz R, Pegg RB. European J Lipid Sci Technol 2008;110(10):865-78.
[14] Anjaneyulu M, Chopra K. Prog Neuropsychopharmacol Biol Psychiatr 2003; 27(6): 1001-5.
[15] Amarowicz R, Estrella I, Hernández T, Dueñas M, Troszyńska A, Kosińska A, et al. Int J Mol Sci 2009;10(12):5513-27.
[16] Zou Y, Chang SK, Gu Y, Qian SY. J Agr Food Chem 2011;59(6):2268-76.
[17] Alcaraz MG, Houli RS. Biochem Pharmacol 1985; 34(14):2477-82.
[18] Toker G, Kupeli E, Memisoglu M, Yesilada E. J Ethnopharmacol 2004; 95(2-3):393-7.
[19] Mehmet O, Yagiz U, Mehmet G. Life Sci 2003; 72:1943-51.
[20] Anjaneyulu M, Chopra K. Prog Neuropsychophamacol Biol Psychiatr 2003;27(6):1001-5
[21] Kaur R, Singh D, Chopra K. J Med Food 2005; 8(4)529-32.
[22] Davidson EM, Coggeshal RE, Carlton SM. Neuroreport 1997;8(4):641-6.
[23] Kupeli E, Tatli LL, Akdemir ZS, Yasilada E. J Ethnopharmacology. 2007; 114(2):234-40.
[24] Hunskaar S, Hole K. Pain 1987; 30(1): 103-14.
[25] Vitale G, Pini LA, Ottani A, Sandrini M. Gen Pharmacol 1998; 31(5): 753-8.
[26] Hunskaar S, Berge OG, Hole K. Pain 1986;25(1): 125-32.