

# Inhibition of Cyclooxygenase Type 1 and 2 Enzyme by Aqueous Extract of *Elaeagnus Angustifolia* in Mice

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## ABSTRACT

**Introduction:** It has been shown that the extract of *Elaeagnus angustifolia* can inhibit inflammation and pain induced by formalin in mice and rats. The aim of the present study is to reach evaluations of possible cellular and molecular mechanisms of *Elaeagnus angustifolia* extract in reducing pain and inflammation through examining the extract ability for inhibition of cyclooxygenase (Cox) type 1 and 2 enzymes and corticosterone release from adrenal glands in mice.

**Methods:** Male Swiss Webster mice were evaluated through the injection of 2  $\mu$ liters to the plantar part of right foot. *Elaeagnus angustifolia* extract was injected to the animals 30 minutes before formalin. In order to evaluate the mechanism of extract, naloxone and memantine were administered intraperitoneally 30 minutes before the extract administration. In separate groups, after injection of extract, blood samples were taken from animals and corticosterone concentrations were measured. In an in vitro study the effect of extract on the activity of cyclooxygenase type 1 and 2 was assessed.

**Results:** the research data showed the ineffectiveness of the extract on acute phase of pain induced by formalin but it completely inhibits the chronic phase. Naloxone and Memantine administration had no effect on the efficacy of extract in the chronic phase. Also the extract administration did not increase the plasma concentration of corticosterone in mice, but in vitro inhibited Cox1 and Cox 2 enzymes.

**Discussion:** These results indicate that *Elaeagnus angustifolia* extract probably reduces pain and inflammation caused by formalin in mice by inhibiting cyclooxygenase type 1 and 2 enzymes.

## 1. Introduction

**P**ain is an unpleasant feeling and a defense mechanism for the body that is considered as a sign of tissue damage and is recog-

nized as a disturbing factor which is intolerable for the individual who reacts against it in order to remove pain stimulus (Coutaux et al., 2005). Different types of strong and damaging stimulants such as heat, low PH and mechanical damage can stimulate the pain sensory

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fibers (Ferreira, 1980). This stimulation increased discharge of afferent pain neurons in proportion to logarithm of stimulus intensity. Following tissue damage, a series of local inflammatory responses along with the pain receptors activation, appeared in the damaged site (Ferreira, 1980). Redness, warmth, swelling and pain are major signs of inflammation (Simmons et al., 2000; Vargaftig and Ferreira, 1981). In reducing and relieving pains\_ which is one of the aims of biological researches \_ chemicals and medicinal plants has long been considered, nevertheless the chemical drugs' great side effects and their high costs caused a tendency toward re-using herbal medicine. Since taking common pain medications leads to many side effects\_ undesirable effects of non-steroidal drugs on digestive system (Chandrasekharan and Simmons, 2004; Ferreira and Nakamura, 1979) or side effects of steroids as delayed wound healing (Millan, 1999; Vargaftig and Ferreira, 1981), increased risk of infection, and edema, and side effects of opioids such as dependency and tolerance (O'Brain et al., 2001), have attracted researchers to achieve a safer analgesic. Iran was not an exception to this tendency (Zargari, 1990) and one of the plants applied most in Iran's traditional medicine is *Elaeagnus angustifolia* (Persian: Senjed) among its health services are the fruit, leaves, flowers and trunk (Zargari, 1990). The fruit is used to eliminate headaches and Rickets, strengthen the stomach and heart and as anti jaundice and the fruit's tea as an analgesic agent to reduce rheumatoid arthritis pain (Alishiri et al., 2007).

Phytochemical studies have shown that aqueous extract of *Elaeagnus angustifolia* fruit contains flavonoids compounds, cardiac glycosides, sitosterol and terpenoid (Dembinska-Migas and Gill, 1973). Researches has shown that flavonoids and sitosterols are responsible for anti-inflammatory and analgesic effects of the plant (Dembinska-Migas and Gill, 1973). Previous studies have demonstrate that the fruit extract of *Elaeagnus angustifolia* has antinociceptive and anti-inflammatory effects in rats (Ahmadiani et al., 2000). In addition, Ramazani and co-workers have shown that the seed of *Elaeagnus angustifolia* also has an antinociceptive effect on mice (Ramezani et al., 2001). Despite of these comprehensive studies, however, the exact mechanism of the action of *Elaeagnus angustifolia* extract is not clear. Since the extract can inhibit phase 2 of formalin test, one can concluded that a peripheral pain pathway(s) can be affected by the extract. Inhibition of inflammation indicated the anti-inflammatory effects of the extract which also is not clear if the extract interacts with cyclooxygenase enzymes or it can influence corticosterone hormone release from adrenal glands. This

study has conducted in order to prove analgesic properties of *Elaeagnus angustifolia* fruit using formalin test in male laboratory mice. In this regard, we use naloxone and memantine for inhibition of opioid and NMDA glutamate receptors respectively for exclusion of possible central pain modulatory pathways involvement in the antinociceptive activity of the extract. In addition, the ability of the extract for induction of corticosterone release and cyclooxygenase inhibition was also examined.

## 2. Methods

### 2.1. Animals

Male Swiss Webster strain of laboratory mice with mean weight of  $25 \pm 2$  g was purchased from Pasteur Institute (Tehran, Iran). The animals were exposed to 12 hours of daylight and fed with standard rat food and tap water (environment temperature,  $23 \pm 2^\circ$  C). In each group of animal experiments, 6 mice were studied. This study was conducted according to standard ethical guidelines and approved by the local ethical committee (The Baqi-yatallah (a.s.) University of Medical Committee on the Use and Care of Animals, 87/381, July 25, 2009). The experiments were done in the light period (10:00 AM-16:00 PM).

### 2.2. Preparation of Extract

100 grams of dry powder of *Elaeagnus angustifolia* fruit was soaked in 1000CC distilled water for 24 hours at  $30^\circ$ C and then the supernatant liquid was placed at the temperature of  $35^\circ$ C to be evaporated in order to obtain the dry extract. 20 grams dry extract was obtained from every 100 grams of powder. This extract was injected to the animal after dissolving in 10 ml saline, intra pretoneally.

### 2.3. Drugs

The following drugs were used in this work: Morphine sulfate (Temad, Iran), dexamethasone, indomethacin, naloxone hydrochloride (Sigma-USA) and memantine bromide (TOCRIS-UK). Drugs were dissolved in saline and injected intrapretoneally to the animals in volumes of 10 ml/kg except for morphine which was given subcutaneously.

### 2.4. Experimental Procedure

To perform formalin test the modified method of Deniss Dubisson was used (Dubisson and Dennis, 1977). For this purpose each animal was placed inside a Plexiglas box with the dimensions of  $30 \times 30 \times 30$  cm

(length × width × height) after injection of formalin in plantar part of right foot. The position of the foot and the way animals responded to formalin injection were evaluated by observers as 0 to 3 score depending on the animal's foot condition. No pains and the normal movement of the animal would score zero, putting foot on the ground rather than putting the body weight on the injected foot (claudicating), score 2 is regarded to the animal avoiding to rub its injected foot with the bottom of box, Score 3 was documented when the rat bit or licked the injected foot from pain. Extract, morphine, dexamethasone and indomethacin were injected to animals 30 minutes before the injection of formalin, while naloxone and memantine were injected to them 30 minutes before (the injection) the extract.

### 2.5. Determining the of Inflammation

For determining the degree of inflammation induced by formalin, Fereidoni et al's method was used (Fereidoni et al., 2000). In this method the animal's left foot was considered as the control foot, in which saline was injected. Animal's right and left foot was separately placed in a container that contained mercury whose exact weight was determined and the mercury weight change was calculated. By calculating the weight change of mercury due to immersion of left foot (control) and right foot (test), foot weight changes were determined after formalin injection and this weight change shifts from the volume change by dividing in to 13.6 (density of mercury).

### 2.6. Experimental Design

#### 2.6.1. Evaluation Of *Elaeagnus Angustifolia* Extracts Analgesia

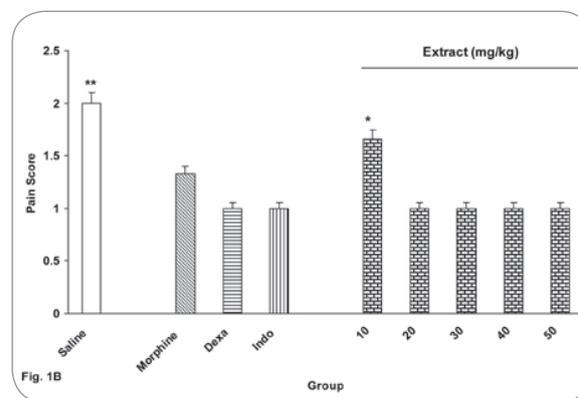
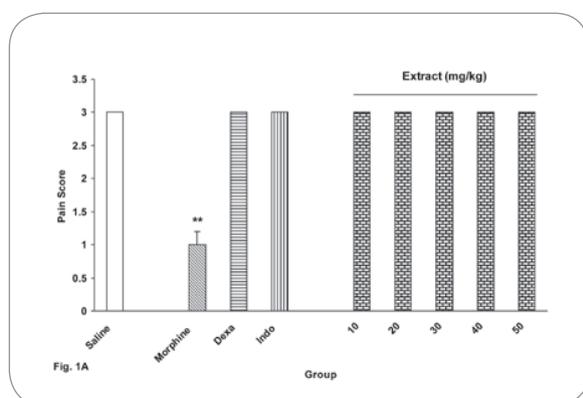
Nine groups of the examining rats (n=6/group) were received either subcutaneous morphine (10 mg/kg), or dexamethasone (10 m6g/kg), indomethacin (10 mg/kg), saline (10 ml/kg), or different doses of *Elaeagnus angustifolia* extract (10, 20, 30, 40 and 50 mg/kg) intraperitonealy 30 min before formalin being injected. Animals' responses to formalin-induced pain were evaluated after formalin injection in to the plantar portion of the right paw.

#### 2.6.2. Evaluation of *Elaeagnus angustifolia* extracts anti-inflammation

Nine groups of the animals (n=6/group) were received either subcutaneous morphine (10 mg/kg), or dexamethasone (10 mg/kg), indomethacin (10 mg/kg), saline (10 ml/kg), or different doses of *Elaeagnus angustifolia* extract (10, 20, 30, 40 and 50 mg/kg) intraperitonealy 30 minutes before formalin. Animals' responses to formalin-induced inflammation were evaluated 30 minutes after formalin injection in to the plantar portion of the right paw.

#### 2.6.3. Study of Opioid or Nmda Glutamate Receptor Inhibition on *Elaeagnus Angustifolia* Extract-Induced Analgesia

Nine groups of rats (n=6/group) were received either memantine (10 mg/kg) + saline (10 ml/kg), naloxone (4 mg/kg) + saline (10 ml/kg), memantine (5 mg/kg) + extract (50 mg/kg), memantine (10 mg/kg) + extract



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**Figure 1A and B.** Effects of *Elaeagnus angustifolia* extract on phase 1 (A) and 2 (B) of formalin test in mice. The extract can not inhibit phase 1 but inhibit phase 2 of the formalin test. Data showed as mean±SEM, for 6 mice, \*P<0.05, \*\*P<0.01 different from experimental groups.

(50 mg/kg), naloxone (1 mg/kg) + Extract (50 mg/kg), naloxone (2 mg/kg) + Extract (50 mg/kg), naloxone (3 mg/kg) + Extract (50 mg/kg), naloxone (4 mg/kg) + Extract (50 mg/kg) intraperitoneally 30 minutes before formalin. Animals' responses to formalin-induced pain were evaluated after formalin injection in to the plantar portion of the right paw.

**2.6.4. Evaluation of *Elaeagnus Angustifolia* Extract on Suppressing Cyclooxygenase Enzyme Type 1 and 2**

Nine groups of under testing rats (n=6/group) were received either subcutaneous morphine (10 mg/kg), or dexamethasone (10 mg/kg), indomethacin (10 mg/kg), saline (10 ml/kg), or different doses of *Elaeagnus an-*

*gustifolia* extract (10, 20, 30, 40 and 50 mg/kg) intraperitoneally 30 minutes before formalin. Animals' serum was collected 30 minutes after formalin injection in to the plantar portion, this serum used for the enzyme inhibition study.

**2.6.5. Evaluation of *Elaeagnus Angustifolia* Extract Ability for Induction of Corticosterone Release from The Adrenal Glands**

Six groups of the animals (n=6/group) were received either saline (10 ml/kg), or different doses of *Elaeagnus angustifolia* extract (10, 20, 30, 40 and 50 mg/kg) intraperitoneally. 30 minutes later, blood sampling from retro-orbital sinus was performed with Plasma corticosterone level determined by ELISA method.

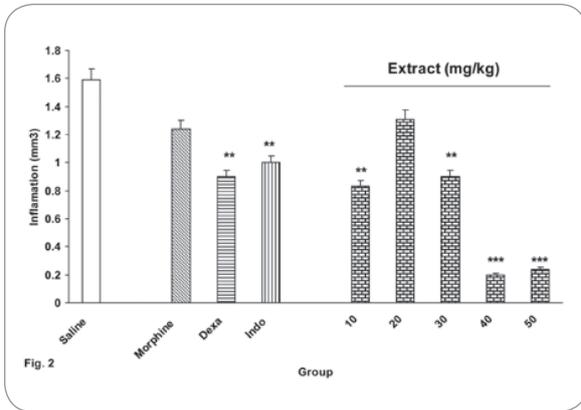


Fig. 2

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**Figure 2.** Effect of *Elaeagnus angustifolia* extract on inflammation induced by formalin in mice. The inflammation was reduced by the extract, dexamethasone, and indomethacin but not morphine. Data showed as mean±SEM, for 6 mice, \*\*P<0.01, \*\*\*P<0.001 different from Saline group.

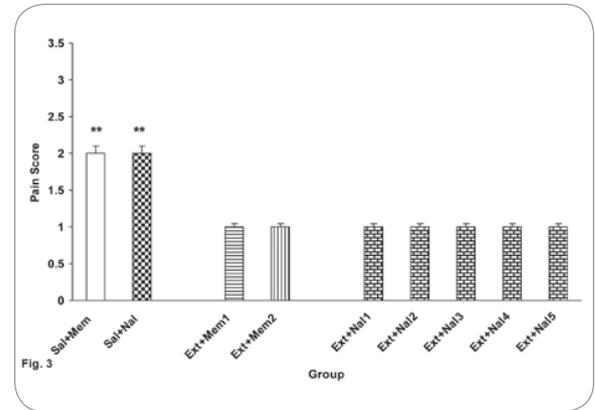


Fig. 3

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**Figure 3.** Effect of opioid and NMDA receptor inhibition on *Elaeagnus angustifolia* extracts effect on phase 2 of formalin pain. Our results indicated that neither naloxone nor memantine did abolish the extract effects. Data showed as mean±SEM, for 6 mice \*\*P<0.01 different from saline treated control group.

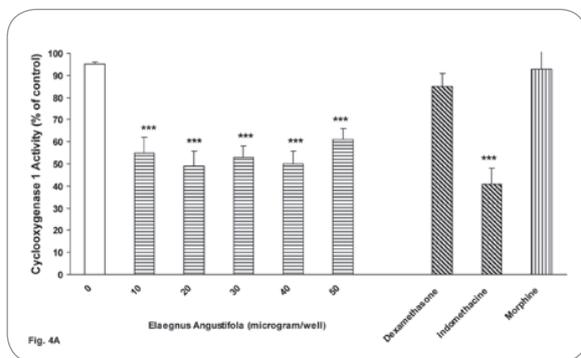


Fig. 4A

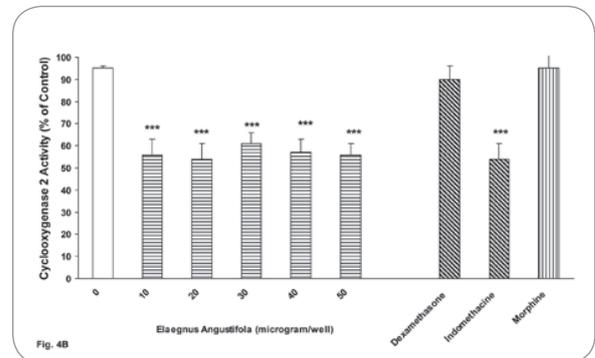
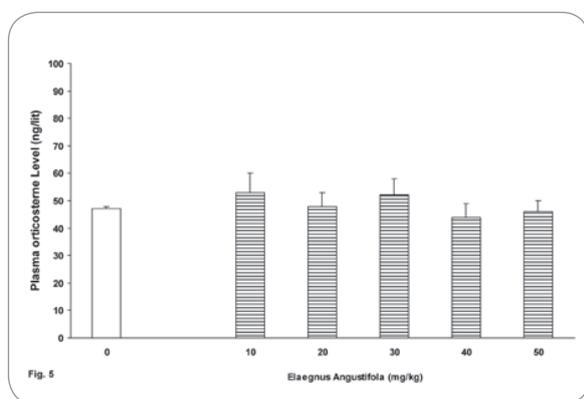


Fig. 4B

**Figure 4A and B.** Inhibition of the enzyme cyclooxygenase type 1 (A) and 2 (B) (Cox 1 and Cox 2) by the extract of *Elaeagnus angustifolia*. The effect is comparable with the effect of indomethacin. Data showed as mean±SEM, for 3×3 well, \*\*\*P<0.001 different from control group.

## 2.7. Determination of Plasma Corticosterone Concentration

30 minutes after injection of extract to the animals eye, 1.0 cc blood was taken from the corner of animals' eye and was added to 1 cc Ependorf pipes containing 0.9 cc sodium citrate solution 2.0. After stirring blood at temperatures of 4 degrees centigrade they were centrifuged at 3000 rpm and the plasma was isolated from blood. The supernatants were used for determination of plasma concentration of corticosterone. To determine the concentration of corticosterone ELISA kit (Rat Corticosterone ELISA kit; EIA-4164; DRG Instruments GmbH, Germany) was used and absorption rate was measured in 450 nm wavelength.



**Figure 5.** Effects of *Elaeagnus angustifolia* extract on corticosterone plasma level in mice. As showed in the figure, the extract can not induce corticosterone level increment in the mice. Data showed as mean $\pm$ SEM, for 6 mice.

## 2.8. Determining Activity of the Enzymes Cyclooxygenase Type 1 and 2 (Cox 1,2)

As mentioned in the previous section, interstitial fluid from the formalin-injected paw was collected using a fine needle (gauge 30) and was added to the ELISA kit which was poured in three well of cyclooxygenase type 1 or 2 enzymes. After 30 minutes incubation at 37 °C, the enzyme product was measured by an ELISA reader in 870 nm.

## 2.9. Statistical Analysis

Data were expressed by Mean  $\pm$  S.E.M. To analyze the data one-way ANOVA was used followed by Tukey post hoc test.  $P < 0.05$  was considered significant.

## 3. Results

### 3.1. Effects of Different Doses of *Elaeagnus Angustifolia* Extract on Formalin-Induced Pain

Different doses of *Elaeagnus angustifolia* extract (10, 20, 30, 40 and 50 mg/kg; i.p.) were injected to the animals 30 minutes before formalin injection accordingly the animals' responses were evaluated 30 minutes later. Data indicated that the extract can not suppress the acute phase of formalin-induced pain [ $F(8, 49) = 6.21$ ,  $P < 0.01$ ] (Fig. 1A). Comparing with morphine, dexamethasone and indomethacin, *Elaeagnus angustifolia* extract demonstrated a comparable effect on suppressing pain in phase 2 in formalin test [ $F(8, 49) = 12.45$ ,  $P < 0.0001$ ] (Fig. 1B).

### 3.2. Effects of *Elaeagnus Angustifolia* Extract on Formalin-Induced Inflammation

The results showed that *Elaeagnus angustifolia* extract (10, 20, 30, 40 and 50 mg/kg, i.p.) can suppress the inflammation induced by formalin in animals [ $F(8, 49) = 9.85$ ,  $P < 0.0001$ ] (Fig. 2).

### 3.3. Effects of Opioid and Glutamate Receptor Inhibition on *Elaeagnus Angustifolia* Extract-Induced Analgesia

The effect of naloxone and memantine on *Elaeagnus angustifolia* extract induced analgesia in phase 2 of formalin test is shown in figure 3. Pretreatment of the animals with naloxone and memantine can not inhibit the extract effects [ $F(8, 49) = 11.23$ ,  $P < 0.001$ ] (Fig. 3).

### 3.4. Effects of *Elaeagnus Angustifolia* Extract on Suppressing Cyclooxygenase Enzyme Type 1 and 2

The obtained results from invitro study showed that, *Elaeagnus angustifolia* extract is able to suppress both cyclooxygenase enzymes type 1 (40-55%) [ $F(8, 49) = 10.75$ ,  $P < 0.0001$ ] (Fig. 4A) and type 2 (50%) [ $F(8, 49) = 17.4$ ,  $P < 0.0001$ ] (Fig. 4B); these effects are comparable to the effects of indomethacin in suppressing the activity of these enzymes.

### 3.5. Effects of Intraperitoneal Administration of *Elaeagnus Angustifolia* Extract on The Plasma Corticosterone Level

The effect of *Elaeagnus angustifolia* extract on plasma corticosterone concentration is shown in Fig.5. The extract did not increase plasma corticosterone level in the experimental groups [ $F(5, 34) = 1.03$ ,  $P > 0.05$ ] (Fig. 5).

#### 4. Discussion

This research, based on the previous researches focused on the effectiveness of *Elaeagnus angustifolia* fruit extract to reduce pain and inflammation caused by administration of formalin (Ahmadiani et al., 2000; Ramezani et al., 2001). Numerous clinical and animal studies showed that using aqueous extract of *Elaeagnus angustifolia* fruit inhibit pain and inflammation in animal models of formalin (Ahmadiani et al., 2000; Ramezani et al., 2001) and in patients with atherosclerosis (Alishiri et al., 2007). Ahmadiani et al specified that extract of *Elaeagnus angustifolia* fruit could inhibit pain and inflammation induced by formalin in male rats and this effect was not associated with inhibition of cyclooxygenase enzyme (Ahmadiani et al., 2000). Researchers have not predicted another mechanism for inhibiting inflammation induced by formalin by *Elaeagnus angustifolia* extract. In the present study, administration of aqueous extract of *Elaeagnus angustifolia* fruit completely inhibits second phase of formalin test (chronic phase), with no significant effect in the acute phase of formalin test. These results were similar to the results of previous researches: Effectiveness of extract was comparable to the ability of indomethacin and dexamethasone to inhibit pain and inflammation caused by formalin injection. Considering the fact that dexamethasone and indomethacin act through two different ways inhibiting the enzymes phospholipase A2 and cyclooxygenase respectively, we evaluated the effect of *Elaeagnus angustifolia* extract on releasing corticosterone hormone as well as effect of extract on cyclooxygenase 1 and 2 enzyme activity. Our tests showed that administration of extract had no effects on plasma corticosterone concentration while inhibited activity of cyclooxygenase 1 and 2 enzymes. Considering these results and previous results we conclude that the effectiveness of the extract in inhibiting pain and inflammation caused by formalin injection is due to its ability to inhibit cyclooxygenase type 1 and 2 enzyme activity and decrease levels of prostaglandin due to tissue damage induced by formalin injection (Chandrasekharan and Simmons, 2004; Coderre et al., 1984; Simmons et al., 2004; Simmons et al., 2000). Researchers showed in several previous experiments that administration of indomethacin and dexamethasone can inhibit inflammation and pain induced by formalin that its mechanism of action as described above is to inhibit the phospholipase A2 enzyme and activity of cyclooxygenase type 1 and 2, respectively (Ferreira et al., 1978; Ferreira and Nakamura, 1979; Hunskaar and Hole, 1987). Considering that *Elaeagnus angustifolia* extract also inhibit cyclo-

oxygenase 1 and 2 on one side, and since no destructive functions on mucous membrane of the gastrointestinal tract by the extract has not reported, it seems that it can be used in chronic pain relief in humans. More over, administration of the opioid receptor antagonist such as naloxone (Abbott et al., 1982), as well as Memantine (NMDA receptors antagonist) (Kavirajan, 2009) did not have the ability to inhibit the extract function. Considering that the effect of extract was on chronic phase induced by formalin and on the other hand the extract performs the function by inhibiting cyclooxygenase enzyme as shown in our study, this lack of efficacy was entirely predictable.

Finally in regard to the effectiveness of *Elaeagnus angustifolia* extract in inhibiting chronic pain and inflammation induced by formalin injection it seems that this extract has a good ability for inhibition of cyclooxygenase type 2 enzymes and also it seems that the extract is a good candidate for becoming a good anti-inflammatory drug.

#### 5. Acknowledgment

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