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# Ellagic acid enhances the anti-nociceptive effects of cyclooxygenase inhibitors in a mouse visceral pain model

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**Introduction:** Combination therapies have long been used to treat painful conditions while reducing side effects. Recently, we have reported the central and peripheral antinociceptive effects of ellagic acid (a polyphenol compound in pomegranates, grapes and different berries). The present study aimed to evaluate the therapeutic potential of combination treatment with ellagic acid (EA) and nonsteroidal anti-inflammatory drugs (NSAIDs) in a visceral model of pain.

**Materials and Methods:** The abdominal writhing test was selected as a model of visceral inflammatory pain. Different doses of EA, indomethacin, celecoxib and acetaminophen alone or in combination with EA were administered.

**Results**: Data showed that EA at doses 1–10 mg/kg i.p. significantly reduced the writhes' number induced by acetic acid in mice. Moreover, intraperitoneal administration of indomethacin at 3, 10 mg/kg, celecoxib at 10, 30 mg/kg, and acetaminophen at 200, 300 mg/kg, significantly reduced the writhing reaction. On the other hand, combination of sub-effective dose of EA (0.3 mg/kg; i.p.) with sub-analgesic doses of indomethacin (0.3, 1 mg/kg; i.p.), celecoxib (1, 3 mg/kg; i.p.) and acetaminophen (60, 100 mg/kg; i.p.) significantly decreased the number of writhes as compared to the *per se* effect.

**Conclusions:** These results indicate that EA markedly potentiates the antinociceptive activity of NSAIDs in visceral pain model. Further, these results suggest that the described combination therapies would be effective as an alternative to conventional NSAIDs and may lower incidence of their adverse effects.

Keywords: Ellagic acid, NSAIDs, Antinociception, Writhing test, Mice

## Introduction

Combination analgesics are often prescribed and used as a substantial portion of the over-the-counter analgesic market. The combination of analgesics is a strategy suggested to obtain one or more therapeutic goals, such as facilitating patient compliance, improving efficacy without increasing adverse effects or decreasing adverse effects without loss of efficacy, and simplifying the prescribing process (Hyllested et al., 2002). There is good evidence that combinations of acetaminophen or non-steroidal anti-inflammatory drugs (NSAIDs) with the other analgesics have an enhanced (synergistic) analgesic effect (Po & Zhang, 1998; Zhang & Po, 1997). However, there are many other analgesic combinations in clinical use with less convincing evidence of additive or synergistic effects of their components.

NSAIDs are widely used in the management of a variety of pain and inflammatory disorders. The molecular target of NSAIDs is the cyclooxygenase (COX) enzymes family. Two isoforms of COX have been identified, namely COX-1 and COX-2, where COX-1 is constitutively expressed in most cells with house-keeping functions and COX-2 is present in low levels in physiological conditions, but rapidly induced by various stimuli such as inflammation (Smith et al., 2000; Svensson & Yaksh, 2002; Warner & Mitchell, 2004). Some NSAIDs such as indomethacin are non-selective inhibitors of COX, while others such as celecoxib are mainly inhibitors of inducible COX-2. However, selective inhibition of COX does not completely account for their efficacy, since various other neurotransmitters are also involved in the antinociceptive

action of NSAIDs (Bjorkman, 1995; Miranda et al., 2001). On the other hand, the acting mechanism of one of the most widely used analgesics, acetaminophen, remains largely unknown and at most the drug is considered to be an atypical NSAID, since it is a weak inhibitor of COXs (Botting, 2003).

Ellagic acid (EA, 2, 3, 7, 8-tetrahydroxybenzopyrano [5, 4, 3-cde] benzopyran-5-10-dione), a polyphenol compound, is one of the active compounds in pomegranate juice, raspberries, grapes, and other foods. It has been reported to show different pharmacological effects including chemoprevention (Townsend & Tew, 2003), inhibition of tumorigenesis (Buniatian, 2003), anti-inflammation and antioxidant (Festa et al., 2001; Lei et al., 2003; Mansouri et al., 2015a), neuroprotection against diabetic neuropathy (Liu et al., 2011), inhibition of anaphylactic reaction *in vivo* and *in vitro* (Choi & Yan, 2009), inhibition of lipopolysaccharide-induced prostaglandin $E_2$ synthesis in human monocytes (Karlsson et al., 2010), inhibition of morphine tolerance to analgesia and dependence (Mansouri et al., 2014a), and also antinociceptive activity in different animal models of pain (Mansouri et al., 2015b; Mansouri et al., 2014b; Mansouri et al., 2013). Additionally, Rogerio et al. (2006) examined the anti-inflammatory and antinociceptive effects of EA in animal models. Their findings showed that EA significantly decreased paw edema, as measured by calipers after an injection of 1% carrageenan, and the number of acid-induced writhing periods in mice. They suggested that the reduction in writhing reactions may be due to cyclooxygenase inhibition or another antinociceptive pathway.

NSAIDs and acetaminophen are drugs widely used to treat moderate to mild pain. However, the use of NSAIDs is limited by ceiling effects and adverse events, especially gastrointestinal and myocardial effects produced by the inhibition of COX-1 and COX-2, respectively (Kulkarni et al., 2000; Ray et al., 2009). Therefore, the purpose of the present study was to investigate the possibility of a potentiation of NSAIDs-induced antinociception by EA in a mouse model of visceral pain.

## Materials and Methods

### Animals

Experiments were conducted using adult male Swiss mice (25–30 g) purchased from the central animal house of the Jundishapur University of Medical Sciences (Ahvaz, Iran). They were housed at 22±2 °C and 12h light/dark cycles (light from 7:00 to 19:00h) with free access to food and water. All animal care and experimental procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. We followed the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983), as well as our institutional guidelines for experiments with animals, designed to avoid suffering and limit the number of animals. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of drug treatments.

### Drugs

Ellagic acid (EA) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The NSAIDs were kindly donated by Darupakhsh Pharmacetical Company (Tehran, Iran). Indomethacin, celecoxib and EA were dissolved in normal saline (0.9%) containing 10% DMSO. Acetaminophen was dissolved in 12.5% of 1, 2-propanediol in normal saline (0.9%). Respective controls received only solvent vehicle. Drug concentrations were freshly prepared in such a way that the necessary dose could be injected in a volume of 5 ml/kg by intraperitoneal route. Doses and drug administration schedules were selected based on the previous reports (Beltz et al., 2008; Rogerio et al., 2006) and our experiences in lab (Mansouri et al., 2014a; Mansouri et al., 2015b; Mansouri et al., 2013).

### Measurement of analgesic activity

Analgesic activity was assessed by the acetic acid abdominal constriction test (writhing test), a chemical visceral inflammatory pain model (Hayashi & Takemori, 1971). This test was selected because it can be a model of clinical relevant intestinal pain in humans (Reichter et al., 2001). All animals were acclimatized to laboratory environment for at least 2 h before testing. Mice were injected i.p. with 10 ml/kg of 0.6% acetic acid according to the method described previously (Mansouri et al., 2015b; Mansouri et al.,2014b; Mansouri et al., 2013). The number of abdominal writhes was counted during a 25-min period, starting 5 min after the administration of acetic acid solution. A writhe was defined as a contraction of the abdominal muscles following by body elongation and hind limbs' extension.

### Experimental design

In order to determine the antinociceptive effect of drugs in mice, increasing doses of EA (0.3, 1, 3 and 10 mg/kg), indomethacin (0.3, 1, 3 and 10 mg/kg), celecoxib (1, 3, 10 and 30 mg/kg) and acetaminophen (60, 100, 200 and 300 mg/kg) were injected to animals 30 min before the acetic acid injection, and writhing behavior was recorded. In order to determine if EA was able to enhance the antinociceptive effects of indomethacin, celecoxib and acetaminophen, increasing ineffective doses of these NSAIDs were co-administered with an ineffective dose of EA (0.3 mg/kg), and writhing behavior was recorded as mentioned above.

## Statistical analysis

The data are shown as the mean±S.E.M. All results were analyzed by analysis of variance (ANOVA) followed by *Tukey's* test. A *p* value of less than 0.05 was considered to be significant. All statistical analyses were carried out using GraphPad software (GraphPad Prism 5, San Diego, CA, USA).

## Results

### Antinociceptive effect EA on writhing test

As shown in Fig. 1, ellagic acid at doses 1, 3, and 10 mg/kg i.p. significantly decreased the number of writhes induced by acetic acid [0.6% (v/v)] in mice ($p < 0.01$).

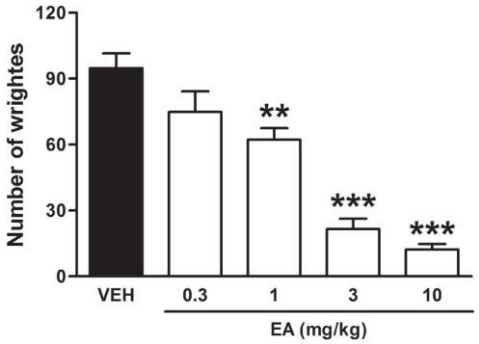


**Figure 1: Antinociceptive effect of ellagic acid (EA, 0.3–10 mg/kg, i.p.) in the mouse writhing test. Data are expressed as mean±SEM (n = 6–8). **p < 0.01 and ***p < 0.001 as compared with vehicle (VEH).**

### Antinociceptive effect of indomethacin alone or combined with EA

The results in Fig. 2A showed that indomethacin at doses 3 and 10 mg/kg i.p. significantly decreased the writhing reaction ($p < 0.05$), but not at doses 0.3 and 1 mg/kg. In addition, EA at non-effective dose (0.3 mg/kg) in combination with non-effective doses (0.3 and 1 mg/kg) of indomethacin dose-dependently decreased the number of writhes in visceral pain model as compared with corresponding control group (Fig. 2B).

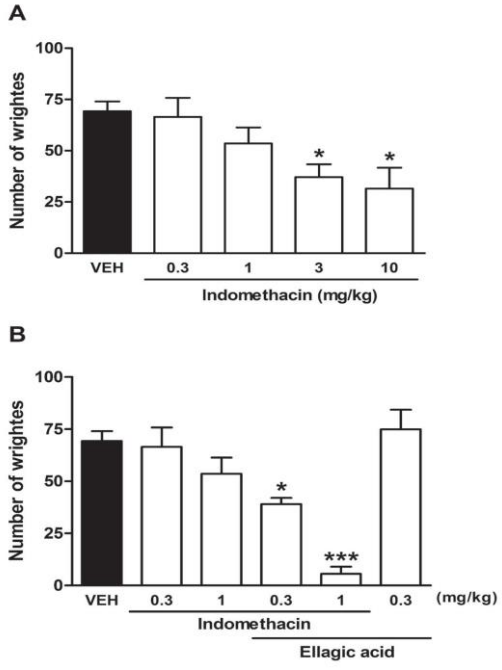


**Figure 2: Antinociceptive effect of indomethacin (0.3–10 mg/kg, i.p.) in the mouse writhing test (A). Potentiation of antinociceptive effect of indomethacin (0.3 and 1 mg/kg, i.p.) by ellagic acid (0.3 mg/kg, i.p.) (B). Data are expressed as mean±SEM (n = 6–8). *p < 0.05 as compared with vehicle (VEH) in panel A, *p < 0.05 and *** p < 0.001 as compared with the *per se* effect in panel B.**

### Antinociceptive effect of celecoxib alone or combined with EA

Administration of celecoxib in mice produced a significant ($p < 0.05$) antinociceptive effect at 10 and 30 mg/kg (Fig. 3A). Moreover, injection of EA at 0.3 mg/kg increased the antinociceptive activity of celecoxib at dose of 3 mg/kg in the test. However, co-administration of EA with celecoxib (1 mg/kg) was not significant (Fig. 3B).

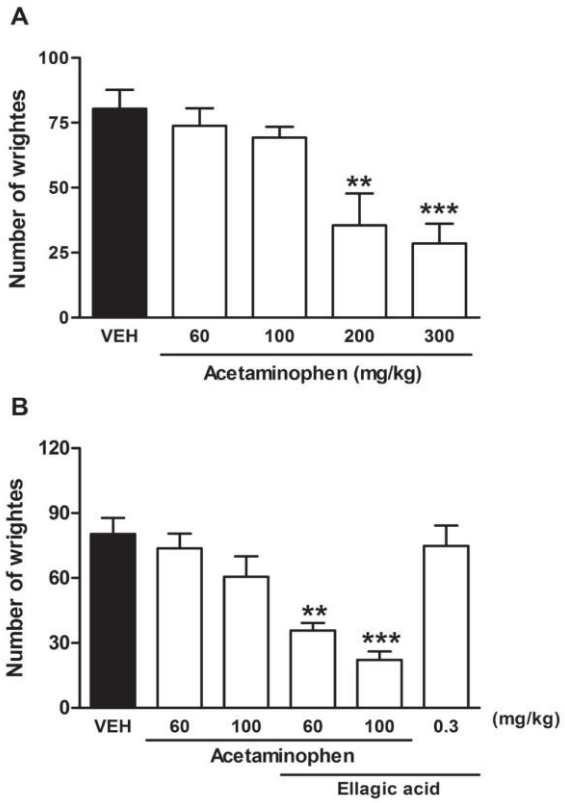


**Figure 3: Antinociceptive effect of celecoxib (1–30 mg/kg, i.p.) in the mouse writhing test (A). Potentiation of antinociceptive effect of celecoxib (1 and 3 mg/kg, i.p.) by ellagic acid (0.3 mg/kg, i.p.) (B). Data are expressed as mean±SEM (n = 6–8). *p < 0.05 as compared with vehicle (VEH) in panel A, *p < 0.05 as compared with the *per se* effect in panel B.**

### Antinociceptive effect of acetaminophen alone or combined with EA

Fig. 4A showed that acetaminophen at doses 200 and 300 mg/kg i.p. significantly decreased the writhing response ($p < 0.05$), but not at 30 and 60 mg/kg. In addition, co-administration of EA (0.3 mg/kg) with non-effective doses of acetaminophen (30, 60 mg/kg; i.p.) produced antinociceptive effect in a dose-dependent manner (Fig. 4B).

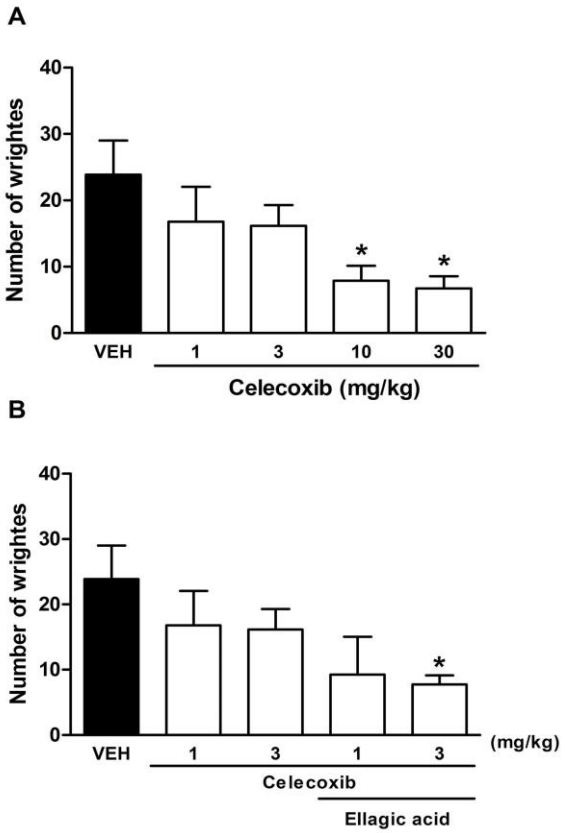


**Figure 4: Antinociceptive effect of acetaminophen (30–300 mg/kg, i.p.) in the mouse writhing test (A). Potentiation of antinociceptive effect of acetaminophen (1 and 3 mg/kg, i.p.) by ellagic acid (0.3 mg/kg, i.p.) (B). Data are expressed as mean±SEM (n = 6–8). **p < 0.01 and ***p < 0.001 as compared with vehicle (VEH) in panel A, **p < 0.01 and ***p < 0.001 as compared with the *per se* effect in panel B.**

## Discussion

In the present study, indomethacin, celecoxib, acetaminophen and ellagic acid alone exhibited comparable dose-dependent antinociceptive activity in the acetic acid-induced writhing test. Also, combination of ellagic acid (EA) with the NSAIDs (indomethacin, celecoxib and acetaminophen) enhanced the antinociception in the test.

In this study, we used the acetic acid writhing test as an inflammatory pain model stimulus for acute nociception. In this test, acetic acid causes tissue damage and releases pain-producing substances, including prostaglandins, which activate peripheral nociceptors on the terminals of sensory nerve fibers. Painful stimuli caused by acetic acid reach higher centers by a number of spinal nerve pathways (Le Bars et al., 2001; Satyanarayana et al., 2004).

A growing pieces of evidence indicated that ellagic acid is effective for altering acute visceral pain (Mansouri et al., 2015b; Mansouri et al., 2014b; Mansouri et al., 2013; Rogerio et al., 2006). Ellagic acid-induced antinociception in the writhing test is also consistent with the ability of this compound to reduce inflammation-induced nociception caused by a variety of inflammatory drugs, such as carrageenan and formalin (Gainok et al., 2011; Mansouri et al., 2014b). The analgesic action of ellagic acid has been explained by the inhibition of cyclooxygenase, which synthesizes prostaglandins at the peripheral cell-damage sites (Mansouri et al., 2015a; Rogerio et al., 2006). Also, in our previous studies, we have shown the central and peripheral antinociceptive effects of EA which were mediated by opioid receptors and L-arginine/NO/cGMP/ATP-sensitive $K^+$ channel pathway using tail-flick, formalin and writhing assays (Ghorbanzadeh et al., 2014; Mansouri et al., 2014b; Mansouri et al., 2013). Furthermore, in our previous study, we observed that EA at doses of 0.3 to 10 mg/kg did not suppress locomotor activities (Mansouri et al., 2013). Hence, EA did not interfere with motor performance at the doses that suppressed acetic acid-induced pain response.

It has been previously reported that systemic administration of NSAIDs such as indomethacin and celecoxib produced antinociceptive activity in different experimental animal models (Schmelzer et al., 2006) and in human volunteers (Burian & Geisslinger, 2005). The results obtained in the present work are in agreement with these findings.

NSAIDs are limited in their use by systemic, gastrointestinal, and cardiovascular side effects (Hinton et al., 2002). So, co-administration of EA with a lower dose of NSAIDs could decrease their requirement doses and leads to a significant decrease in the side effect profile of these drugs. In the writhing test, acetic acid activates peripheral nociceptors on the sensory nerve fibers by releasing pro-inflammatory substances (Satyanarayana et al., 2004) and NSAIDs could attenuate pro-inflammatory substances synthesis by inhibiting COX-1 and COX-2. However, the analgesic effect of NSAIDs may not simply reflect a common mechanism of action, namely inhibition of prostaglandin biosynthesis (Chandrasekharan et al., 2002; Warner & Mitchell, 2004) and may act by mechanisms that involve actions on spinal and supraspinal nociceptive transmission. For example, it was reported that the active mechanism of NSAIDs may involve the release of acetylcholine in the spinal cord (Pinardi et al., 2003) and supraspinal serotonin (Guerinot et al., 1974).

Our present data demonstrated that EA combined with indomethacin or celecoxib produced an enhanced analgesic effect. In agreement to our findings, Gainok et al. (2011) showed that EA may enhance the antinociceptive effect of ketorolac in paw pressure test. Although the exact mode of action of EA is not clear, we previously demonstrated the central and peripheral antinociceptive effect of EA induced by systemic and local administration (Mansouri et al., 2013). Furthermore, we suggested the involvement of opioid system and NO-cGMP pathway in the antinociceptive effect of ellagic acid (Mansouri et al., 2015a; Mansouri et al., 2015b; Mansouri et al., 2014b; Mansouri et al., 2013). In addition, research suggests that EA inhibits a number of cell-signaling pathways that are important to tumor growth, including inflammatory signaling such as tumor necrosis factor α–induced COX-2 protein expression (Adams et al., 2006). Despite the above findings, it seems that the observed potentiation of the

combination therapy could be involved in the neurotransmitter systems or the inhibitory action of EA on inflammatory mediators. Previous studies have demonstrated the antinociceptive potentiation between ellagic acid and several drug classes such as venlafaxine (Mansouri et al., 2015b), carbamazepin (Naghizadeh et al., 2015), sildenafil (Mansouri et al., 2014c), a known cyclooxygenase inhibitor ketorolac (Corbett et al., 2010), and morphine (Mansouri et al., 2014a).

Furthermore, the co-administration of acetaminophen and EA showed similar result. Acetaminophen is often classified as an NSAID, because it possesses analgesic activity against pain of mild to moderate severity but has few anti-inflammatory properties and exerts its analgesic effect *via* a central action. Several clinical studies (Bippi & Frohich, 1990; Seppala et al., 1990) have failed to show a reduction in peripheral prostaglandins in response to acetaminophen. In fact, acetaminophen is only a weak inhibitor of peripheral prostaglandin synthesis (Brune et al., 1991), whereas it interferes with prostaglandin synthesis in the central nervous system (Clissold, 1986; Flower & Vane, 1972). A mechanism has been suggested for acetaminophen-induced analgesia in the mouse abdominal constriction test, namely that acetaminophen produces a central action paralleled by a reduction in brain prostaglandin $E_2$ concentrations (Ayoub et al., 2006).

According to these findings and our previous observation, the fact that EA potentiates the antinociceptive effect of acetaminophen could be interpreted as an indication of the activation of different and complementary central and peripheral mechanisms. However, the exact mechanism of these interactions needs to be addressed in future investigations.

## Conclusion

In conclusion, these results demonstrate that systemic administration of ellagic acid, a commonly used herbal supplement, may enhance the antinociceptive effects of cyclooxygenase inhibitors such as indomethacin, celecoxib and acetaminophen in the visceral model of pain. Furthermore, these results suggested that the combinations of ellagic acid and NSAIDs would be effective as an alternative to conventional NSAIDs treatment in the inflammatory nociceptive conditions and may lower incidence of adverse effects, as the use of combinations can reduce the total amount of any one drug required for antinociception.

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## Conflict of interests

The authors declare that there are no conflicts of interest regarding this manuscript.

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