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Effect of curcumin on formalin-induced muscle pain in male rats: role of local cyclooxygenase system

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Abstract

Investigating the mechanisms responsible for pain processing of natural and synthetic chemical compounds is necessary to optimize pain management. Curcumin (Cur), the active ingredient of turmeric, exhibits potent analgesic and anti-inflammatory properties by employing multiple mechanisms at the local peripheral, spinal and supra-spinal levels. This study was aimed to investigate the effect of oral administration of Cur on muscle pain induced by intramuscular (IM) injection of formalin. To explore the possible local mechanisms, a cyclooxygenase (COX) inhibitor, diclofenac (Dic) and a COX product, prostaglandin E_2 (PGE_2), were applied. The IM injection of formalin (25.00 µL, 2.50%) into the gastrocnemius muscle induced two distinct phases of hind leg flinching. A short-lasting (10 min) hind leg lifting was observed following IM injection of PGE_2 (2 µg kg^{-1}, 25.00 µL). Oral administration of Cur (25.00 and 100 mg kg^{-1}) and IM injection of 40.00 µg kg^{-1} Dic attenuated formalin and PGE2 induced nociceptive behaviors. Contra-lateral IM injection of Dic did not change muscle pain induced by ipsilateral IM injection of formalin and PGE_2. The second phase of formalin induced flinching as well as PGE_2 evoked lifting were more suppressed when 40.00 µg kg^{-1} Dic and 100 mg kg^{-1} Cur were used together. Locomotor activity was not changed by the above-mentioned treatments. It was concluded that the reducing effect of muscle pain of Cur might be related to the local inhibition of COX.

Introduction

Noxious chemical, mechanical and thermal stimuli cause muscle pain by stimulating nociceptors located in the walls of arterioles and connective tissue of skeletal muscles.[1] Spinal and medullary dorsal horn receive and transmit muscle nociception through spinal and trigeminal ascending pain pathways for supra-spinal processing.[2] Inflammatory mediators including prostaglandin E_2 (PGE_2), bradykinin, serotonin and tumor necrosis factor-alpha (TNF-α) play prominent roles in the chemical stimulation of muscle nociceptors.[3,4] In this context, injection of TNF-α into the gastrocnemius muscle caused pain and increased calcitonin gene related peptide and nerve growth factor expression in the muscle.[5] In addition, anterior tibial muscle injection of PGE_2 potentiated protons (pH 6.00 and 6.50) induced muscle nociception in humans.[6] Due to the involvement of nociceptive and inflammatory mediators, injections of acidic saline, hypertonic saline and formalin into the masseter and gastrocnemius muscles have been provided muscle pain models in humans and rodents.[7,8]

Curcumin (Cur; 1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione), as an active ingredient of turmeric (*Curcuma long*), is a natural lipophilic polyphenol with anti-inflammatory, antioxidant, anti-diabetic and anticancer properties.[9] Curcumin is considered a potent pain reliever by employing multiple mechanisms. For example, in a diabetic neuropathic pain model, oral administration of Cur attenuated mechanical allodynia and heat hyperalgesia by reducing spinal cord dorsal horn expression of TNF-α and TNF-α receptor 1.[10] In addition, Cur reduced pain in both phases of formalin induced orofacial pain by decreasing the amplitude of acid-sensing ion channel currents in trigeminal ganglion neurons.[11] In cancer induced bone pain, naloxone as a non-selective antagonist of opioid receptors blocked the antinociceptive effect of Cur indicating the involvement of opioid system.[12]

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Although the mechanisms of muscle pain are poorly understood, the use of non-steroidal anti-inflammatory drugs alone or in combination with other analgesics such as muscle relaxants, tricyclic antidepressants and anticonvulsants is the first choice for alleviating muscle pain.[13,14] Due to the side effects and drug tolerance of the aforementioned drug categories, searching and replacing effective treatments is one of the optimal goals of pain management.[14] In this regard, medicinal plants and their bioactive chemical compounds are widely used as optimal pain management.[15] Accordingly, the present study was planned to investigate the effects of *per os* (PO) of Cur on formalin-induced muscle pain. To find the possible local mechanism of Cur action, intramuscular (IM) injections of diclofenac (Dic) as a cyclooxygenase (COX) inhibitor and PGE_2 as a COX product into the gastrocnemius muscle were included in the present research.

Materials and Methods

Animals. In this research, adult male Wistar rats (210 - 230 g) were used. The animals were maintained in standard breeding conditions temperature: 22.00 ± 0.50 ˚C, humidity: 60.00 - 70.00% and 12 hr light-dark cycles. Food and water were ad libitum. Pain-related behavior recording was done between 10.00 AM - 15.00 PM. The study protocol was approved by Veterinary Ethics Committee of Urmia University Faculty of Veterinary Medicine (Ethical code: IR-UU-AEC-3/49).

Drugs. Curcumin and formalin solution 37.00% were purchased from Merck (Darmstadt, Germany). The PGE_2 and Dic sodium were purchased from Sigma-Aldrich, St. Louis, USA. Curcumin was suspended in distilled water (DW). Diclofenac and PGE_2 were dissolved in normal saline and ethanol 3.00%, respectively. Formalin solution was diluted by normal saline. Chemical solutions were prepared 30 min before use.

Study protocol. Oral administration of Cur and IM injection of Dic were done 45 and 1 min before induction of muscle pain by IM injection of formalin and PGE_2, respectively. To confirm the local effect, Dic was also injected into the contralateral gastrocnemius muscle. The observers were blinded to the study protocol.

Animal grouping. In the present study, 96 rats were divided into 16 groups of six animal each as fallows: Group 1 received IM injection of normal saline. Group 2 received IM injection of formalin. Group 3 received IM injection of ethanol. Group 4 received IM injection of PGE_2. Group 5 - 7 were treated with PO administrations of 6.25, 25.00 and 100 mg kg^{-1} Cur, respectively, before IM injection of formalin. Groups 8 - 10 were treated with PO administrations of 6.25, 25.00 and 100 mg kg^{-1} Cur, respectively, before IM injection of PGE_2. Groups 11 and 12 received IM injections 40.00 µg kg^{-1} Dic into the ipsilateral (IL) and contra-lateral (CL) gastrocnemius muscles, respectively, before IM injection of formalin. Groups 13 and 14 received IM injections 40.00 µg kg^{-1} Dic into the IL and CL gastrocnemius muscles, respectively, before IM injection of PGE_2. Group 15 was treated with PO administration of 100 mg kg^{-1} Cur and IM injection of 40.00 µg kg^{-1} Dic, before IM injection of formalin. Group 16 was treated with PO administration of 100 mg kg^{-1} Cur and IM injection of 40.00 µg kg^{-1} Dic, before IM injection of PGE_2. Chemical compound doses used here were in accordance with previous studies,[16-18] and also our preliminary experiments.

The PO and IM administrations. A suspension solution of Cur in DW was prepared and PO administered by gavage at a constant volume of 3.00 mL kg^{-1}.[19] Normal saline, ethanol, Dic, PGE_2 and formalin were IM injected in the belly of gastrocnemius muscle using a 30-guage injection needle at a constant volume 25.00 µL.

Muscle pain. Muscle pain was induced by IM injection of formalin and PGE_2 into the gastrocnemius muscle.[18,19] A 30-min adaptation period was considered after placing of the animal in a clear Plexiglas box (30.00 × 30.00× 30.00 cm). After IM injection of formalin (50.00 µL, 2.50%), hind leg flinching number was counted in five min intervals for a 60-min period. Hind leg lifting duration was measured after IM injection of PGE_2 every two min for 20 min.

Locomotor activity test. Locomotor activity was recorded using an electronic activity box (Borj Sanat, Tehran, Iran). The animals were carefully put in the center of the activity box and then the number of beam breaks caused by animal movement were recorded in a five min session as a measure of locomotor activity.

Statistical analysis. Data were statistically analyzed using GraphPad Prism (version 8.2; GraphPad Software Inc., San Diego, USA). The time-point results were analyzed using two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test. Flinching number of the first and second phases, lifting duration and beam break number were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Data are presented as mean ± SEM. A p value smaller than 0.05 was considered for all results.

Results

Regarding hind leg flinching number (Fig. 1A), two-way repeated measures ANOVA revealed significant differences among treatments ($F_{(1,120)} = 495.2$, $p < 0.0001$), times ($F_{(11,120)} = 31.40$, $p < 0.0001$) and interactions ($F_{(11,120)} = 29.73$, $p < 0.0001$). Subsequent analysis with Bonferroni's test expressed that IM injection of normal saline produced a weak flinching response, whereas, IM injection of formalin caused more flinching in the first and 5^{th} - 9^{th} 5-min intervals. Considering hind leg lifting (Fig. 1B), two-way repeated measures ANOVA revealed significant differences between treatments ($F_{(1,100)} = 278.6$,

$p < 0.0001$), times ($F_{(9,100)} = 42.02$, $p < 0.0001$) and interactions ($F_{(9,100)} = 35.57$, $p < 0.0001$). Further analysis with Bonferroni's test indicated that IM injection of ethanol did not induce hind leg lifting, whereas, IM injection of PGE_2 at a dose of 2.00 µg kg^{-1} caused more hind leg lifting in 1st - 5th 2-min intervals.

Oral administration of Cur at doses of 25.00 and 100 mg kg^{-1}, but not at a dose of 6.25 mg kg^{-1}, significantly decreased the formalin-induced hind leg flinching number at the first ($F(3,20) = 31.19$, $p < 0.0001$, Fig. 2A) and second ($F(3,20) = 47.09$, $p < 0.0001$, Fig. 2A) phases. The PGE_2-induced hind leg lifting duration was also decreased by 25.00 and 100 mg kg^{-1} ($F(3,20) = 37.20$, $p < 0.0001$, Fig. 2B), but not by 6.25 mg kg^{-1} Cur.

The IL, IM injection of 40.00 µg kg^{-1} Dic with no effect on the first phase (Fig. 3A), significantly decreased the second phase ($F_{(2,15)} = 36.98$, $p < 0.0001$, Fig. 3A) of formalin-induced hind leg flinching number as well as PGE_2-provoked lifting duration ($F_{(2,15)} = 45.07$, $p < 0.0001$, Fig. 3B). Formalin-induced hind leg flinching (Fig. 3A) and PGE_2-induced hind leg lifting (Fig. 3B) were not altered by CL IM injection of 40.00 µg kg^{-1} Dic.

The reducing effect of 100 mg kg^{-1} Cur on the first phase of hind leg flinching was not changed by 40.00 µg kg^{-1} Dic (Fig. 4A). Diclofenac (40.00 µg kg^{-1}) enhanced the reducing effect of 100 mg kg^{-1} Cur on both the second phase of formalin-induced hind leg flinching ($F_{(3,20)} = 68.81$, $p < 0.0001$, Fig. 4A) and PGE_2 induced lifting ($F_{(3,20)} = 104.5$, $p < 0.0001$, Fig. 4B).

With no significant differences, beam break numbers following IM injection of normal saline, ethanol, Dic and PGE_2 and PO administration of DW and Cur and Cur before Dic at the doses mentioned in animal grouping were 109.67 ± 5.42, 98.83 ± 5.58, 101.67 ± 4.47, 97.16 ± 6.02, 102.34 ± 4.33, 105.56 ± 6.15, 108.23 ± 4.74, 96.85 ± 3.87, 108.74 ± 5.57, respectively.

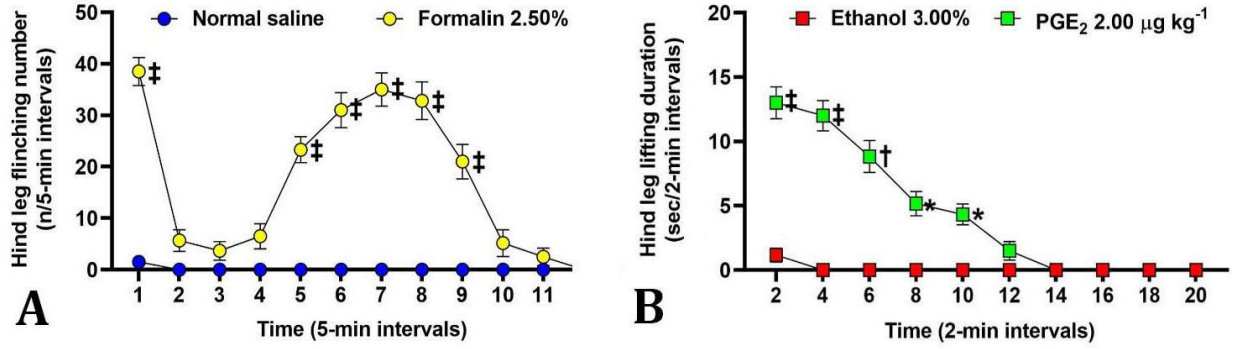

Fig. 1. A) Time dependent hind leg flinching following intramuscular (IM) injection of normal saline and formalin 2.50% and **B)** Time-dependent hind leg lifting after IM injection of ethanol and 2.00 µg kg^{-1} prostaglandin E_2 (PGE_2). Normal saline and ethanol were IM administered one min before IM injection of formalin and PGE_2, respectively. Values from each group are the mean ± SEM (n = 6). * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$ in comparison with corresponding control group.

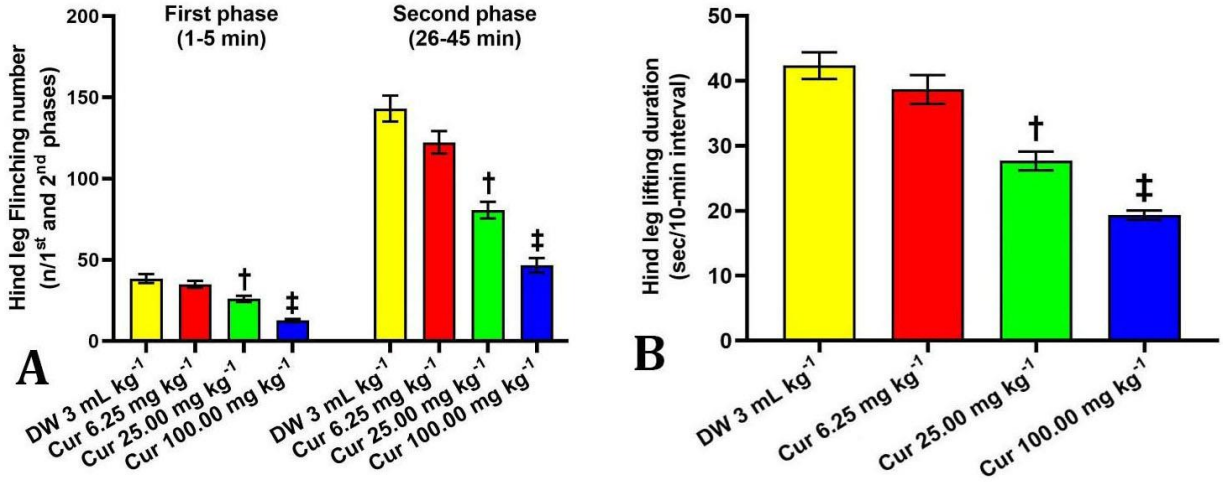

Fig. 2. The effects of *per os* (PO) administration of distilled water (DW) and curcumin (Cur) at doses of 6.25, 25.00 and 100.00 mg kg^{-1} on **A)** first (1 - 5 min) and second (26 - 45 min) phases of hind leg flinching induced by intramuscular (IM) injection of formalin, and **B)** Hind leg lifting induced by IM injection of prostaglandin E_2 (PGE_2). The Cur was PO administered 45 min before IM injections of formalin and PGE_2. Values from each group are the mean ± SEM (n = 6). † $p < 0.01$ and ‡ $p < 0.001$ in comparison with corresponding DW group.

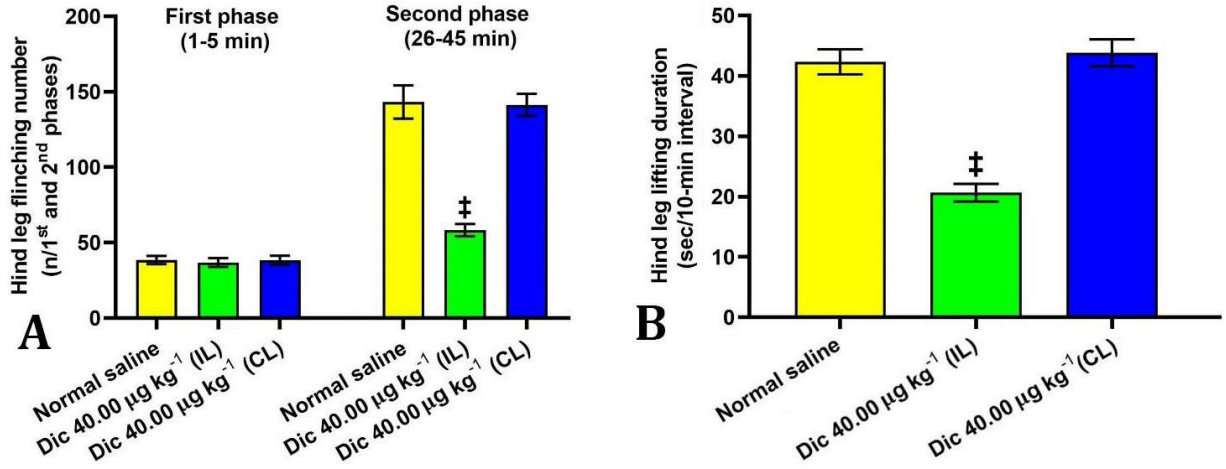

Fig. 3. The effects of ipsilateral (IL) and contralateral (CL) intramuscular (IM) injection of normal saline and 40.00 µg kg^{-1} diclofenac (Dic) on **A)** Formalin induced flinching number, and **B)** The PGE_2-induced hind leg lifting duration. The Dic was IM administered one min before IM injections of formalin and PGE_2. Values from each group are the mean ± SEM (n = 6). † $p < 0.01$ and ‡ $p < 0.001$ in comparison with normal saline group.

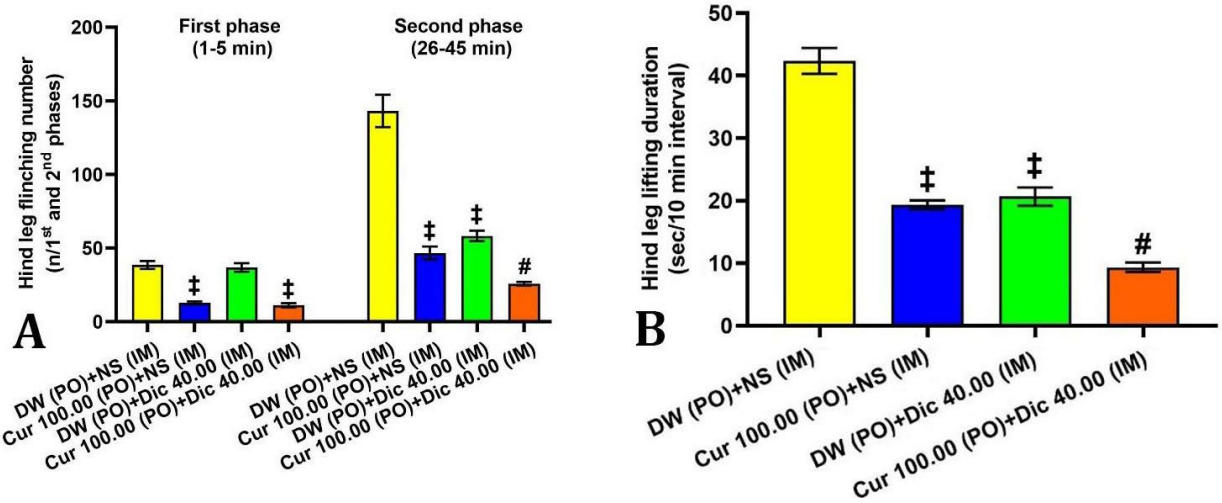

Fig. 4. The effects of IM injection of 40.00 µg kg^{-1} diclofenac (Dic) after *per os* (PO) administration of distilled water (DW) and 100 mg kg^{-1} curcumin (Cur) on the **A)** First (1 - 5 min) and second (26 - 45 min) phases of hind leg flinching induced by intramuscular (IM) injection of formalin, and **B)** Hind leg lifting evoked by IM injection of prostaglandin E_2 (PGE_2). The Cur and Dic were PO and IM administered 45 and one min before IM injection of formalin and PGE_2. Values from each group are the mean ± SEM (n = 6). ‡ $p < 0.001$ and # $p < 0.0001$ in comparison with corresponding DW group.

Discussion

In the current study, after IM injection of formalin and PGE2, flinching and lifting of the hind leg were appeared, respectively, which are consistent with the previous findings.[18-20] Although the role of local mediators in producing muscle pain after IM injection of formalin is not fully understood, the involvement of TNF-α, nerve growth factor, bradykinin, serotonin and PGE_2 have been considered.[3,4,21] In this context, injections of formalin and PGE_2 into the gastrocnemius muscle have been used to investigate hyperalgesia mechanisms in humans and rodents.[18,21,22]

Our present study results indicated that PO administration of Cur attenuated formalin and PGE_2 induced pain-related behaviors. Although the analgesic effect of Cur on formalin-induced muscle pain has not been reported so far, oral administration of Cur at doses of 31 and 100 mg/kg produced a pain-relieving effect in the hind paw plantar surface injection of formalin induced pain.[23] It has been also found that intraperitoneal injection of Cur 15 min before formalin injection into the vibrissa pad causes reduction of facial grooming.[24] Moreover, it has been reported that prior intra-plantar (IPL) injection of naloxone, AM 251 (a specific antagonist of CB_1 receptors) and AM 630 (a CB_2 receptor antagonist) inhibits the

antinociceptive effect of subsequent IPL injection of Cur dissolved in 3.00% DMSO in a rat model of carrageenan induced hyperalgesia.[25] Curcumin seems to have beneficial effects in the treatment of a wide range of pain states by employing different types of local mechanisms including inhibition of a number of pro-inflammatory mediators, inhibition of oxidative stress and COX-2, down-regulation of calcium channels like transient receptor potential and inhibition of apoptosis.[26]

In the present study, IM injection of Dic into the IL, but not CL gastrocnemius suppressed inflammatory pain induced by formalin and PGE_2. This means the possible contribution of local COX enzymes in formalin-induced inflammatory muscle pain. Diclofenac, a benzene acetic acid derivative, is used as a potent analgesic and anti-inflammatory agent by inhibition of both COX-1 and COX-2 enzymes.[27] Oral and topical applications of Dic have been frequently used to treat of acute and chronic pain such as musculoskeletal pain states.[28] In this context, topical application of Dic has been used to treat acute musculoskeletal pain states such as myofascial pain syndrome.[29] Although the involvement of the COX, especially COX-2 has been determined to some extent in the pain and inflammation caused by formalin injection into the hind paw and the upper lip,[30,31] no evidence of this issue has been raised in gastrocnemius muscle. In other skeletal muscles such as the masseter muscle it was found that Dic injection into the muscle reduced mechanical hyperalgesia and muscle PGE_2 concentration caused by TNF injection into the muscle.[32]

In the current study, the attenuating effects of Cur on PGE_2 induced nociception and the second phase of formalin-induced pain were enhanced by Dic. This showed that the analgesic effect of Cur was probably done by employing local anti-inflammatory mechanisms such as inhibition of COX activity in the muscle tissue. Although there is no report showing the systemic effect of Cur on the local action of Dic in formalin induced muscle inflammatory pain, a synergistic effect between systemically applied Cur and Dic has been reported in the second phase of pain induced by IPL injection of formalin in rats.[23] A synergistic effect between systemic Cur and Dic has also been found in the model of inflammatory pain induced by formalin injection into the vibrissa pad in rats.[24] Curcumin and Dic in a combination treatment enhanced the bioavailability of Cur, produced anti-inflammatory effect and alleviated arthritis symptoms in streptococcal wall model in mice when compared to alone use of Cur and Dic.[33] The results of the present study suggested that the systemic Cur produced more beneficial effect when used together with local application of Dic.

Current study results expressed that locomotor activity was not influenced by the abovementioned treatments. This result confirmed previous findings in which systemic and oral administration of 25.00 - 200 mg kg^{-1} Cur and local application of 100 - 200 µg kg^{-1} Dic did not cause any alterations in motor behavior in the open field test.[31,34] When examining the analgesic effect of a synthetic or natural chemical compound, their effect on motor activity should be measured in order to understand the hypo, and hypermotor functions because both of which might affect the analgesic activity.[35]

In conclusion, the results of the present study indicated oral administration of Cur and IM injection of Dic attenuated formalin and PGE_2 induced muscle pain. Documented analgesic effects were observed when PO administration of Cur was used together with IM injection of Dic. Analgesic effect of Cur might be mediated by inhibition of local peripheral COX activity.

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Conflict of interest

No financial or other conflicts of interest are declared by the authors.

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