

## Effects of intrathecal and intracerebroventricular microinjection of kaempferol on pain: possible mechanisms of action

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### Abstract

**Background and purpose:** Kaempferol (KM), a flavonoid, has an anti-inflammatory and anticancer effect and prevents many metabolic diseases. Nonetheless, very few studies have been done on the antinociceptive effects of KM. This research aimed at assessing the involvement of opioids, gamma-aminobutyric acid (GABA) receptors, and inflammatory mediators in the antinociceptive effects of KM in male Wistar rats.

**Experimental approach:** The intracerebroventricular and/or intrathecal administration of the compounds was done for examining their central impacts on the thermal and chemical pain by the tail-flick and formalin paw tests. For assessing the role of opioid and GABA receptors in the possible antinociceptive effects of KM, several antagonists were used. Also, a rotarod test was carried out for assessing motor performance.

**Findings/Results:** The intracerebroventricular and/or intrathecal microinjections of KM (40 µg/rat) had partially antinociceptive effects in the tail-flick test in rats ( $P < 0.05$ ). In the formalin paw model, the intrathecal microinjection of KM had antinociceptive effects in phase 1 (20 and 40 µg/rat;  $P < 0.05$  and  $P < 0.01$ , respectively) and phase 2 (20 and 40 µg/rat;  $P < 0.01$  and  $P < 0.001$ , respectively). Using naloxonazine and/or bicuculline approved the involvement of opioid and GABA receptors in the central antinociceptive effects of KM, respectively. Moreover, KM reduced the expression levels of caspase 6, interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and interleukin-6. The antinociceptive effects of KM were not linked to variations in the locomotor activity.

**Conclusion and implications:** It can be concluded that KM has remarkable antinociceptive effects at a spinal level, which is associated with the presence of the inflammatory state. These impacts were undetectable following injections in the lateral ventricle. The possible mechanisms of KM antinociception are possibly linked to various modulatory pathways, including opioid and GABA receptors.

**Keywords:** Antinociception; Kaempferol; Pain; Spinal cord; Supraspinal.

### INTRODUCTION

Pain is a major healthcare problem worldwide (1). According to some estimations, 1 of 5 adults experiences pain each year. In addition to decreasing life quality, the pain increases the cost of general health leading to economic losses to society (2). Regrettably, the existing analgesic drugs have several side effects and are either too potent or too weak. Consequently, it is necessary to look for novel analgesic compounds as therapeutic alternatives (3,4).

Flavonoids are polyphenolic plant compounds frequently found in fruits and they are categorized into flavones, isoflavones, flavonols, flavanones, flavanols, and anthocyanins groups (5,6). According to the epidemiological and pharmacological studies, flavonoids have several effects, like antioxidative, anticancer, and antiviral impacts (7).

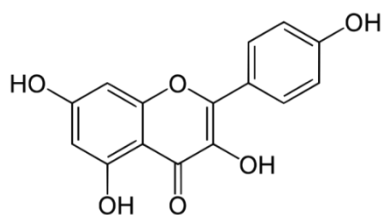
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**Fig. 1.** Chemical structure of kaempferol (C<sub>15</sub>H<sub>10</sub>O<sub>6</sub>).

Kaempferol (KM; Fig. 1) as a flavonoid is available in several edible plants (tea leaves, broccoli, endive, tomato, strawberries, and grapes) or botanicals often applied in traditional medicine (*Moringa oleifera* and *Sophora japonica*). An association has been reported between eating foods rich in KM and a decreased chance of many diseases including cancer and cardiovascular diseases. According to several preclinical investigations, KM and its some glycosides exhibit diverse pharmacological activities, such as antioxidant, antimicrobial, neuroprotective, anti-osteoporotic, anxiolytic, and anti-allergic effects (8-10). Bian *et al.* showed that KM suppresses several pathways associated with releasing inflammatory mediators from lipopolysaccharide-related intestinal microvascular endothelial cells in rats (11). Furthermore, Qian *et al.* have shown that KM decreases K63-linked polyubiquitination for inhibiting nuclear factor- $\kappa$ B and inflammatory reactions in acute lung trauma in mice (12). According to these results, KM has an anti-inflammatory effect. It has been reported that pro-inflammatory cytokines, like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and IL-6 are effective in the process of pain, and up-regulation of peripheral and central amounts of these factors is seen in several pain patterns. Nevertheless, limited reports have been issued on the antinociceptive effect of KM (13-15), and none of the mentioned studies assessed antinociceptive effects of KM administered intracerebroventricularly (ICV) or intrathecally (IT) in rats. Accordingly, the current study was done to investigate the antinociceptive activity caused by ICV or IT administration of KM in thermal and chemical models of pain in rats, examine pro-inflammatory cytokines as well as opioid and gamma-aminobutyric acid (GABA) mechanisms associated with the

antinociceptive activity due to KM in the formalin paw (FP) test, and assess the acute and chronic toxicity caused by KM in rats to indicate the safety of KM.

## MATERIALS AND METHODS

### Animals

Adult male Wistar rats weighing 180-250 g were provided from Pasteur Institute of Iran (Tehran, I.R. Iran). They were maintained under 12/12-h light/dark, humidity 50  $\pm$  5%, at 22  $\pm$  2  $^{\circ}$ C condition, and water and food were freely accessible. The experiments were carried out in the light period (10:00-16:00). The examiner was blinded to the treatments, and animals were classified in random to different groups (n = 6 per group). The ethics committee of Hamadan University of Medical Sciences, confirmed the testing and animal care procedures (Ethic No. IR.UMSHA.REC.1397.139). The experiments were carried out following the National Institutes of Health Publication No. 85-23, which was revised in 1985. Reducing the number of rats used and alleviating their suffering were considered.

### Drugs and chemicals

KM, purity  $\geq$  97% (10, 20, and 40  $\mu$ g/rat; product code: 60010); naloxone hydrochloride (NLX; 5  $\mu$ g/rat, product code: N7758); diclofenac sodium (Dic; 6.25  $\mu$ g/rat, product code: S0765000), naloxonazine, a  $\mu$ -opioid receptor antagonist, (Nax; 5  $\mu$ g/rat, product code: N176); nor-binaltorphimine,  $\kappa$ -opioid receptor antagonist (Nbt; 5  $\mu$ g/rat, product code: N1771); naltrindole,  $\delta$ -opioid receptor antagonist (Ntd; 5  $\mu$ g/rat, product code: N115); bicuculline, the antagonist of GABA<sub>A</sub> receptors (Bic; 1  $\mu$ g, product code: 14340); saclofen, the antagonist of GABA<sub>B</sub> (Sac; 2  $\mu$ g, product code: S166), tetrahydropyridin-4-yl methylphosphinic acid, the antagonist of GABA<sub>C</sub> (TPMPA; 5  $\mu$ g/rat, product code: T200), dimethyl sulfoxide (DMSO; product code: D8418), formaldehyde solution (product code: F8775), ketamine hydrochloride (product code: 1356009), and xylazine (product code: X1251) were all purchased from Sigma-Aldrich Corporation, USA. Morphine hydrochloride

(5 µg/rat, product code: Cat. No. 5158) was purchased from Tocris Corporation, USA. After dissolving the KM in DMSO, it was diluted with normal saline before administration. Normal saline was applied for dilution of Dic and morphine as positive controls. The selected concentrations of drugs were based on the previous literature and the outcomes of our initial experiments (10,16-21). Furthermore, because normal saline or DMSO administrations have shown the same results without any acute or chronic toxicity, we decided to use DMSO as a control or vehicle in all experiments.

### ***ICV cannulation***

First, the animals were anesthetized by intraperitoneal injection of ketamine combined with xylazine (80 and 10 mg/kg, respectively). Then, a stainless steel cannula (21-gauge; 12 mm) was implanted into the right lateral ventricle for ICV injection. According to the previous literature (22,23), the stereotaxic coordinates were as follows: 1.5 mm right lateral, 0.8 mm posterior, and 4.0 mm ventral toward the bregma. After securing the cannula using dental cement, fastening was done using stainless steel screws (fastened to the skull) followed by sealing through a stainless steel wire for inhibiting occlusion. After seven days of recovery, the rats were housed separately before the experiments. The drug solution was added to the injection cannula (29-gauge 15 mm), which was attached to a Hamilton syringe (10-µL) by a polyethylene-20 catheter, and then added to the guide cannula, that extended 1 mm past the cannula tip. DMSO (5%, 10 µL) was used in combination with Tween<sup>®</sup> 20 and saline (0.99% NaCl) at 5:5:90 (v/v) ratio (24). Within 20 min, DMSO or KM (10, 20, and 40 µg/rat) were administered.

### ***IT catheter insertion***

Based on the method described in the previous literature (25,26), lumbar catheters were inserted for intrathecal administration. In the intrathecal space, a stretched polyethylene-10 catheter (10-µL void volume; 8.5 cm) was placed under anesthesia by intraperitoneal xylazine and ketamine (10 and 80 mg/kg, respectively) injection. Then, by making an

incision in the atlantooccipital membrane, the catheter was passed caudally toward the rostral end of the lumbar enlargement. After a five-day recovery, the rats were housed separately.

### ***KM acute and chronic toxic effects***

For assessing KM safety, its acute and chronic toxicity was measured. Abnormal behaviors and anomalies regarding diet, feces, body weight, hair, activities, and gross anatomy in animals following the IT and/or ICV injection of the vehicle or KM (10, 20, or 40 mg/kg) within the initial three days and after 14 days were studied.

### ***Tail-flick test***

The antinociceptive reaction to the thermal stimulus was measured by the tail-flick (TF) test (PANLAB 7160, Spain). The rats were restrictively held with the tail on a slot (adjustable width) using a groove to ensure accurate placement in the TF apparatus for radiant thermal stimulation of the dorsal surface of the rear. The stimulus intensity was fixed to force the rats to flick their tail in 2-4 s as the baseline TF latency. The gap between the start of heat exposure and the tail withdrawal was calculated to determine the TF latency. The TF latency was assessed before and 30, 60, 90, and 120 min following the central administration of the drugs. The cut-off time was adjusted at 10 s for minimizing tissue injury (26,27).

### ***FP test***

The FP test was done based on the Yin *et al.* approach (28). In brief, the rats were put separately in glass beakers allowed for adaptation within 30 min prior to the test. The vehicle or KM (10, 20, and 40 µg/rat) were injected 15 min before the formalin injection. Morphine (5 µg/rat) or Dic (6.25 µg/rat) were injected 15 and 30 min, in the respective order, before the formalin administration as a positive control. Afterward, formalin solution injection (5%; 25 µL) was done in the plantar region of the right hind paws of rats followed by putting them quickly in the beakers and a mirror was placed under the beaker for observing their paws. The duration of biting or licking the paw that was subjected to injection was determined using a stopwatch each 5 min and was considered as a sign of nociception.

### **Role of the opioid system**

The opioid system potential effect on the antinociception activities of KM was explored using NLX (5 µg/rat), and selective opioid receptors, Nax, Nbt, and Ntd, before either morphine (5 µg/rat) or KM (40 µg/rat). Next, the rat's paw was subjected to formalin (5%, 25 µL) injection 15 min following morphine or KM administration using the FP model (29,30).

### **Role of the GABA system**

The potential effect of the GABA system on the antinociceptive activities of KM was investigated through the injection of Bic, Sac, and TPMPA, as selective GABA receptor antagonists, before administrating KM (40 µg/rat). Then, formalin injection (5%, 25 µL) was done into the paw 15 min following administrating KM in the FP model (25,31).

### **Rotarod test**

According to the literature (32,33), an accelerating rotarod (47700; Ugo Basile) was used to examine the impact of KM on motor function. Time to falling was calculated in seconds by placing the normal rats on a rotarod with an increase in velocity (between 4 and 40 rpm in 5 min), which forced them to move onward to avoid falling. The animals' baseline reaction was determined on experiment day and the impacts of ICV and IT injection of DMSO and KM on motor performance were studied, repetitively for 120 min after injections.

### **Caspase 6/TNF- $\alpha$ pathway, IL-1 $\beta$ , and IL-6 effects in the spinal cord**

For assessing potential effects of caspases 6 (CASP6)/TNF- $\alpha$  pathway, IL-1 $\beta$ , and IL-6 in the spinal cord on the analgesic activities triggered *via* KM, pretreatment of the rats (15 min) using the vehicle or KM (10, 20, or 40 µg/rat) was done. Then, 60 min following the injection of formalin, the lumbar enlargement of the spinal cord was immediately isolated to investigate the proCASP6 (total CASP6), a-CASP6 (CASP6 active type), TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 concentrations.

### **Western blotting**

When the rats were deeply anesthetized by sevoflurane, their lumbosacral enlargement was immediately isolated and maintained at -80 °C until the next analyses. Lysis buffer plus protease inhibitors were used for homogenizing the tissue samples, which then underwent incubation on ice (30 min) and centrifugation (13,000 rpm, 20 min), followed by collecting the supernatants. The bicinchoninic acid protein assay kit (Sigma-Aldrich, USA) was applied to determine protein concentrations, and the identical level of each protein was isolated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8%) following the producer's guidelines. Next, the transfer of the proteins into polyvinylidene fluoride membranes was done. Then, they were subjected to blockage using 5% skim milk in phosphate-buffered saline by 0.1% Tween<sup>®</sup> 20 in 2 h at room temperature. Following blockage, these membranes underwent incubation for a night (4 °C) using CASP6-antibody at 1:1,000 dilution (Cell Signaling Technology; USA), aCASP6 at a dilution of 1:2,000 (active/cleaved, Imgenex; USA), TNF- $\alpha$  at a dilution of 1:800, IL-1 $\beta$  at a dilution of 1:1000, IL-6 at a dilution of 1:1000, or  $\beta$ -actin at a dilution of 1:4000 (the last three were obtained from Abcam; USA). We washed the membranes using phosphate-buffered saline with Tween<sup>®</sup> buffer followed by incubation by horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution (Jackson, USA) within 2 h at room temperature. Then, an enhanced chemiluminescence system (Santa Cruz Biotechnology, USA) was used to identify immune complexes. The intensity of the blots was evaluated with the ImageJ program (National Institutes of Health, Maryland, USA).

### **Statistical analysis**

Data analysis was carried out by Statistical Package for the Social Sciences (SPSS) 16.0 and the values are introduced as mean  $\pm$  SD. The obtained findings were compared through one-way or two-way analysis of variance (ANOVA) (repeated measures) and the Bonferroni post-hoc test. Statistical significance was set at a *P* value of smaller than 5%.

## RESULTS

**Acute and chronic toxicity of KM**

Administering KM (IT or ICV) caused no abnormal behavior or abnormality affecting animals' nutrition, hair, body weight, feces, activities, or gross anatomy. There was not a significant difference through the first three days or after 14 days, indicating the lack of acute or chronic toxicity caused by KM (data not shown).

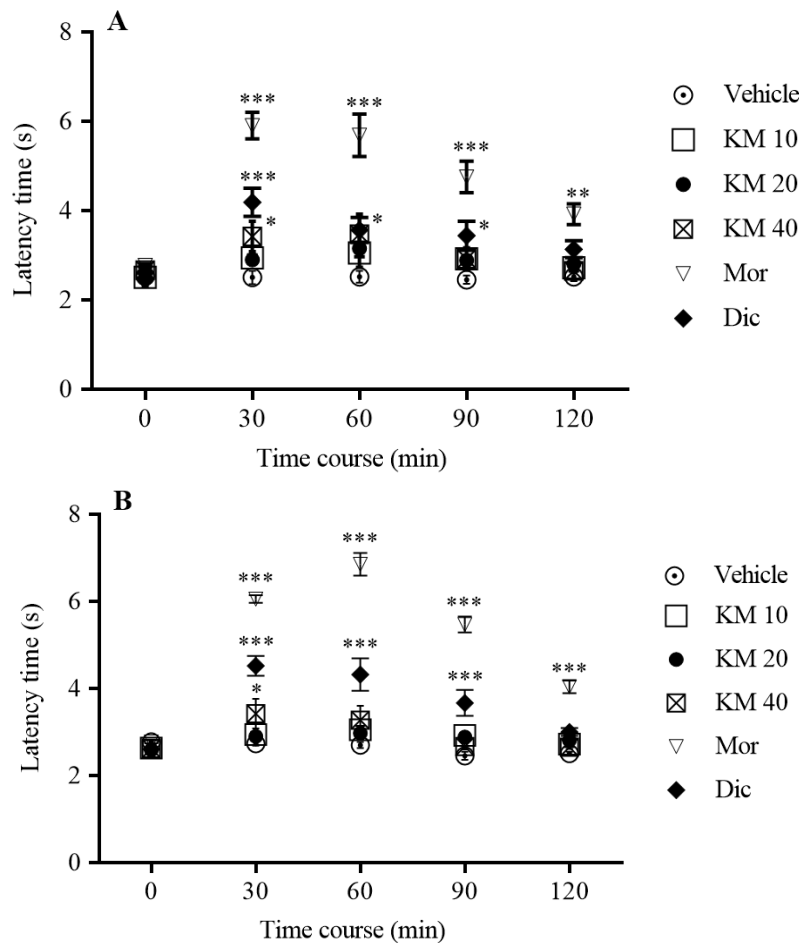
**TF reaction time against IT and ICV administration of KM**

Figure 2A indicates that in comparison with the control group, KM (40  $\mu\text{g}/\text{rat}$ , IT) demonstrated significant antinociceptive activity at 30 and 60 min ( $P < 0.05$ ). The microinjection of either morphine or Dic showed the antinociceptive effects at 30 min

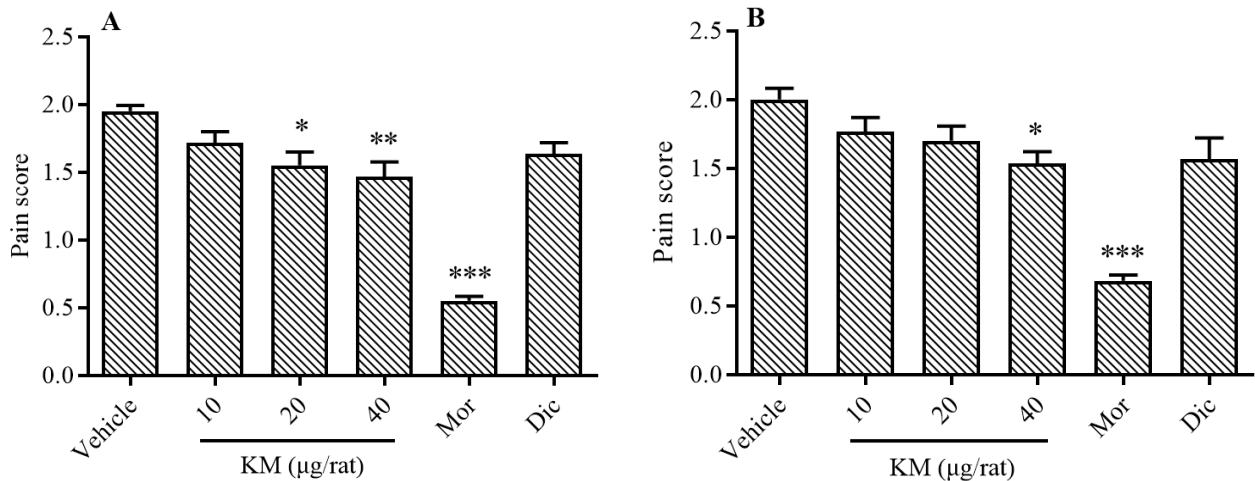
( $P < 0.001$ ). According to the results, Fig. 2B indicates that in comparison with the control group, KM (40  $\mu\text{g}/\text{rat}$ , ICV) demonstrated antinociceptive activity only at 30 min ( $P < 0.05$ ). In addition, microinjection of either morphine or Dic showed the antinociceptive effects at 30 min ( $P < 0.001$ ).

**Impact of KM on FP test in rats.**

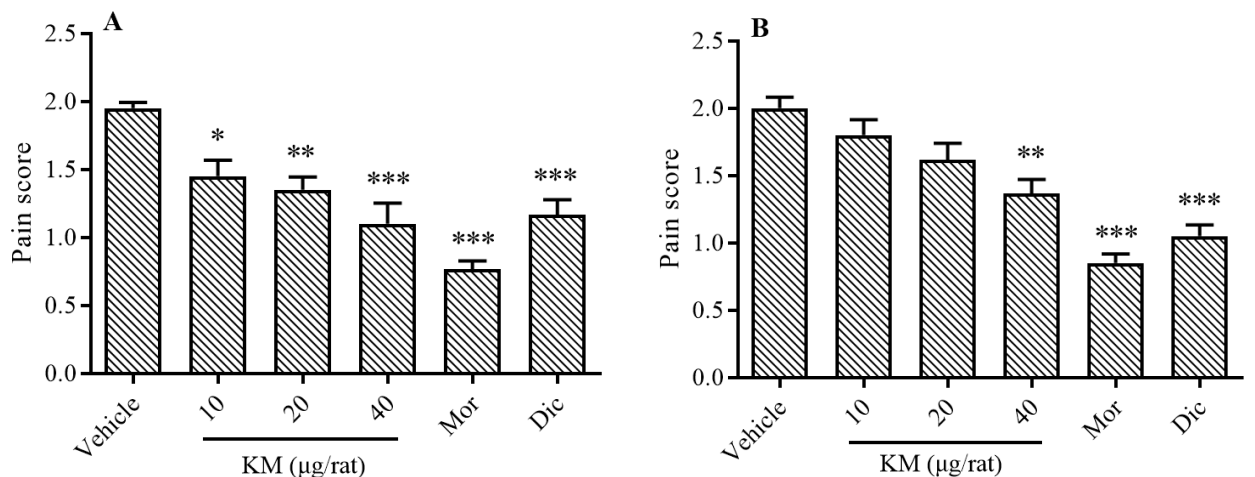
Based on Fig. 3A, the results revealed that KM (20 and 40  $\mu\text{g}/\text{rat}$ , IT) significantly reduced the flinching/shaking behavior in phase 1 of the test ( $P < 0.05$  and  $P < 0.01$ , respectively). Also, in this phase, the microinjection of morphine significantly reduced the flinching/shaking behavior ( $P < 0.001$ ) in rats. According to Fig. 3B, the results revealed that KM only at 40  $\mu\text{g}/\text{rat}$  (ICV) could decrease the flinching/shaking behavior in phase 1 ( $P < 0.05$ ).



**Fig. 2.** Alteration in latency time in the tail-flick reaction against (A) intrathecal and (B) intracerebroventricular administration of KM and DMSO (vehicle/control). Animals were injected with KM (10, 20, or 40  $\mu\text{g}/\text{rat}$ ), Mor, Di, or control/vehicle (5  $\mu\text{L}$ ) and the tail-flick reactions were evaluated at 0, 30, 60, 90, and 120 min following the treatments ( $n = 6$ ). Values are introduced as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  significantly differ from the vehicle group. Dic, Diclofenac; DMSO, dimethyl sulfoxide; KM; kaempferol; Mor, morphine.



**Fig. 3.** Pain score after (A) intrathecal or (B) intracerebroventricular injections of KM at 10, 20, or 40 µg/rat, Mor, and Dic in phase 1 of the formalin test (n = 6). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  indicate significant differences against the vehicle group (DMSO). Dic, Diclofenac; DMSO, dimethyl sulfoxide; KM; kaempferol; Mor, morphine.



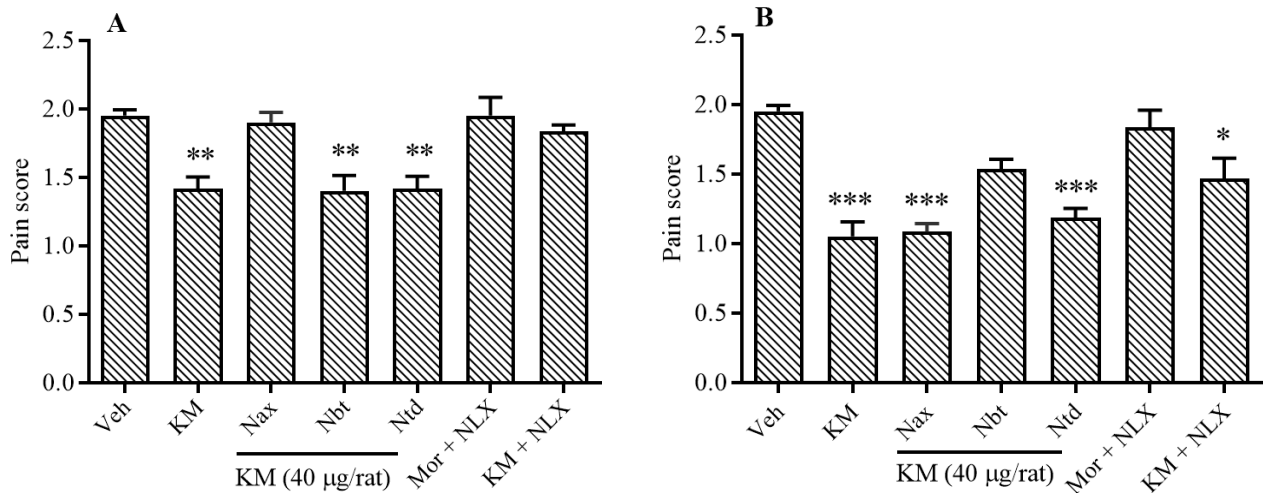
**Fig. 4.** Pain score after (A) intrathecal or (B) intracerebroventricular injections of KM at 10, 20, or 40 µg/rat, Mor, and Dic in phase 2 of the formalin test (n = 6). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  significantly different from the vehicle group (DMSO). Dic, Diclofenac; DMSO, dimethyl sulfoxide; KM; kaempferol; Mor, morphine.

In addition, in this phase, the microinjection of morphine significantly reduced the flinching/shaking behavior ( $P < 0.001$ ) in rats. Based on Fig. 4A, all doses of KM (IT) could decrease the flinching/shaking behavior in phase 2 of the test. Moreover, in this phase, microinjection of Dic, in contrast to phase 1, significantly reduced the flinching/shaking behavior ( $P < 0.001$ ) in rats. According to Fig. 4B, only a dose of 40 µg/rat of KM (ICV) reduced the flinching/shaking behavior in phase 2 of the test ( $P < 0.01$ ). In addition, either morphine or Dic microinjection significantly lowered the flinching/shaking behavior

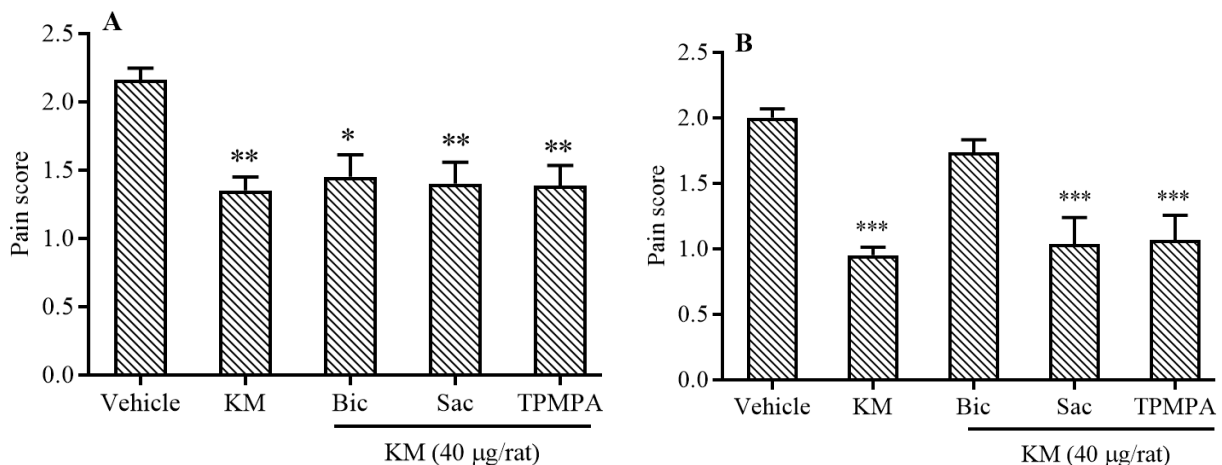
( $P < 0.001$ ) in rats.

***Opioid system involvement by the antinociceptive activity of KM***

According to Fig. 5A, the results revealed that the antinociceptive activities of KM (40 µg/rat, IT) in phase 1 of the formalin tests were completely antagonized by Nax (5 µg/rat, IT) and/or NLX (5 µg/rat, IT). Also, the obtained data indicated that in phase 2 of the FP test, Nbt (5 µg/rat, IT) entirely prevented the antinociceptive activities of KM. Also, NLX slightly prevented the KM antinociceptive effects ( $P < 0.05$ ; Fig. 5B).



**Fig. 5.** Opioid system involvement in the KM analgesic activity at 40  $\mu\text{g}/\text{rat}$  after intrathecal injection in (A) phase 1 and (B) phase 2 of the formalin test;  $n = 6$ . \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  significantly different from the vehicle group (dimethyl sulfoxide). KM; kaempferol; Nax, Naloxonazine; Nbt, nor-binaltorphimine; Ntd, naltrindole, NLX, naloxone



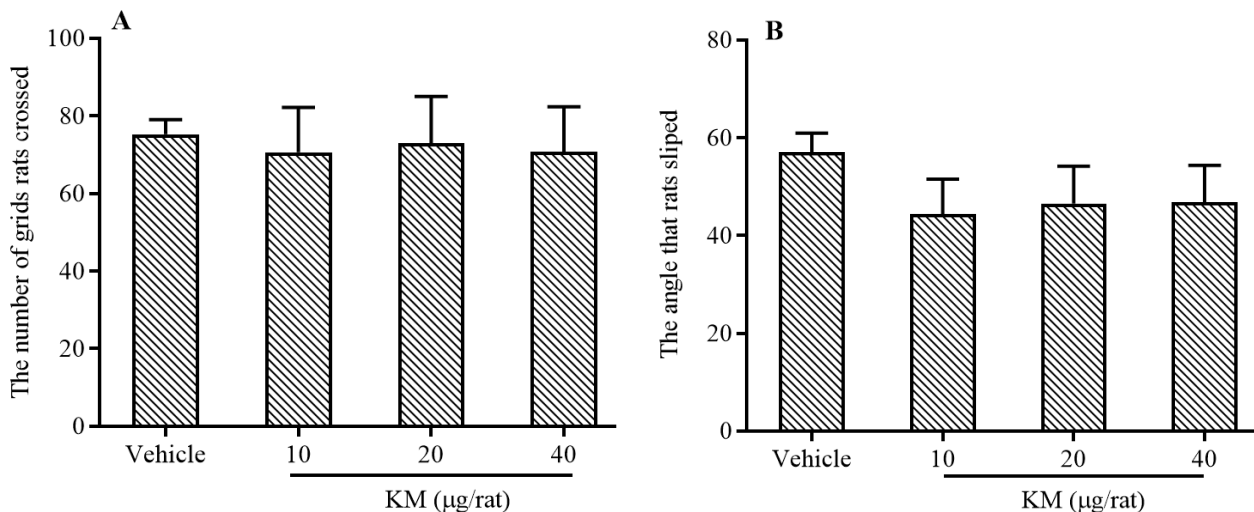
**Fig. 6.** GABA system role in the KM antinociceptive activity at 40  $\mu\text{g}/\text{rat}$  after intrathecal injection in (A) phase 1 and (B) phase 2 of the formalin test;  $n = 6$ . Bic, an antagonist of GABA<sub>A</sub> receptors; Sac, an antagonist of GABA<sub>B</sub> receptors; TPMPA, an antagonist of GABA<sub>C</sub> receptors. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  indicate significant differences in comparison with the vehicle group (dimethyl sulfoxide). Bic, Bicculline; GABA, gamma-aminobutyric acid; KM; kaempferol; Sac, saclofen; TPMPA, (tetrahydropyridin-4-yl) methylphosphinic acid.

### Role of the GABA system in the antinociceptive activity of KM

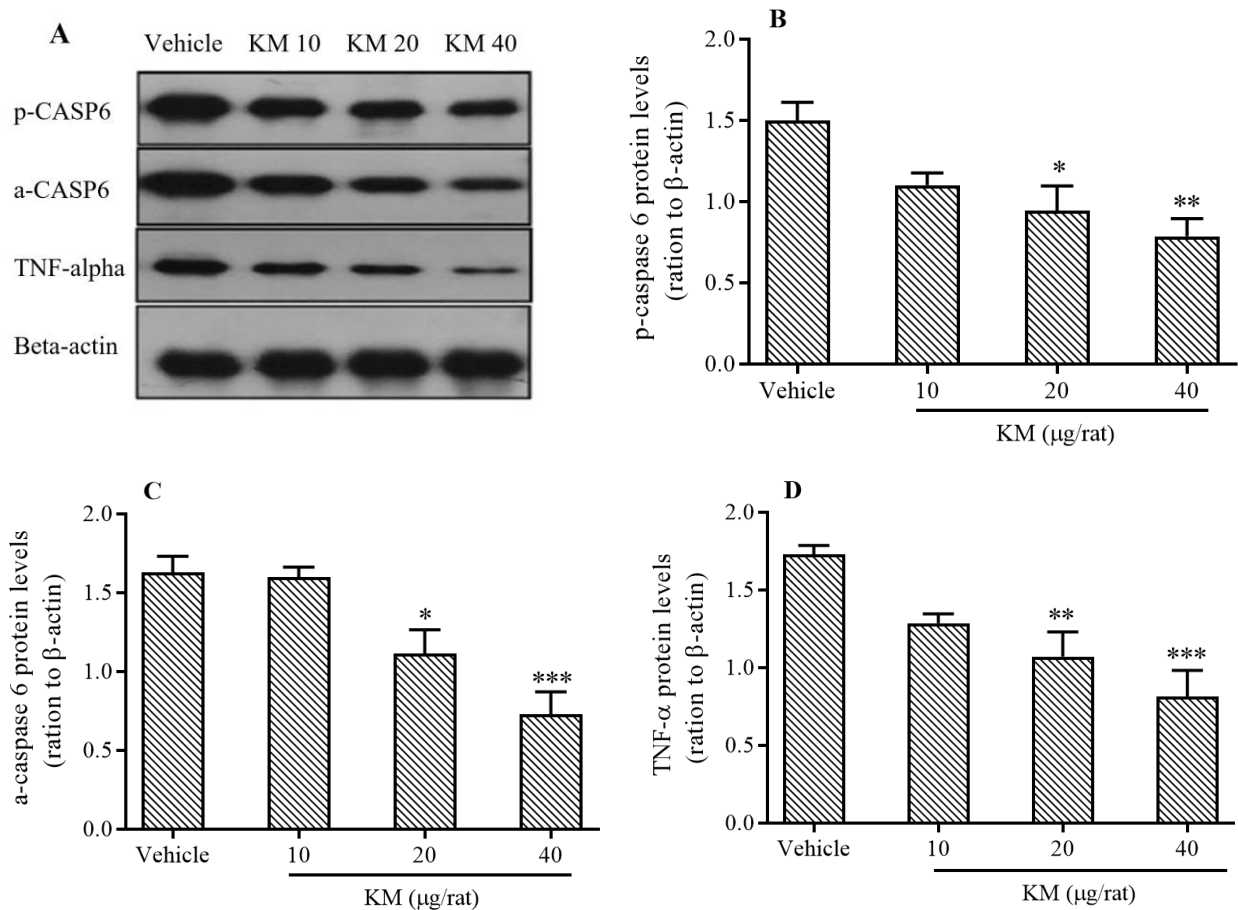
According to Fig. 6A, the results revealed that the antinociceptive activities of KM (40  $\mu\text{g}/\text{rat}$ , IT) in phase 1 of FP test ( $P < 0.05$ ) were partially antagonized by Bic (1  $\mu\text{g}/\text{rat}$ , IT). Also, the results indicated that the antinociceptive activities of KM (40  $\mu\text{g}/\text{rat}$ , IT) in phase 2 of the test were completely antagonized by Bic (1  $\mu\text{g}/\text{rat}$ , IT; Fig. 6B).

### Impact of KM on locomotor function and motor reactions

To eliminate the possible less important KM-related antinociceptive effects compared with sedative/muscle relaxant effects, the KM impact on locomotor function and motor reactions was explored. The dose, at which KM could exert intense antinociceptive effects could not significantly ( $P > 0.05$ ) affect the locomotor function and motor reactions than the control group (Fig. 7).

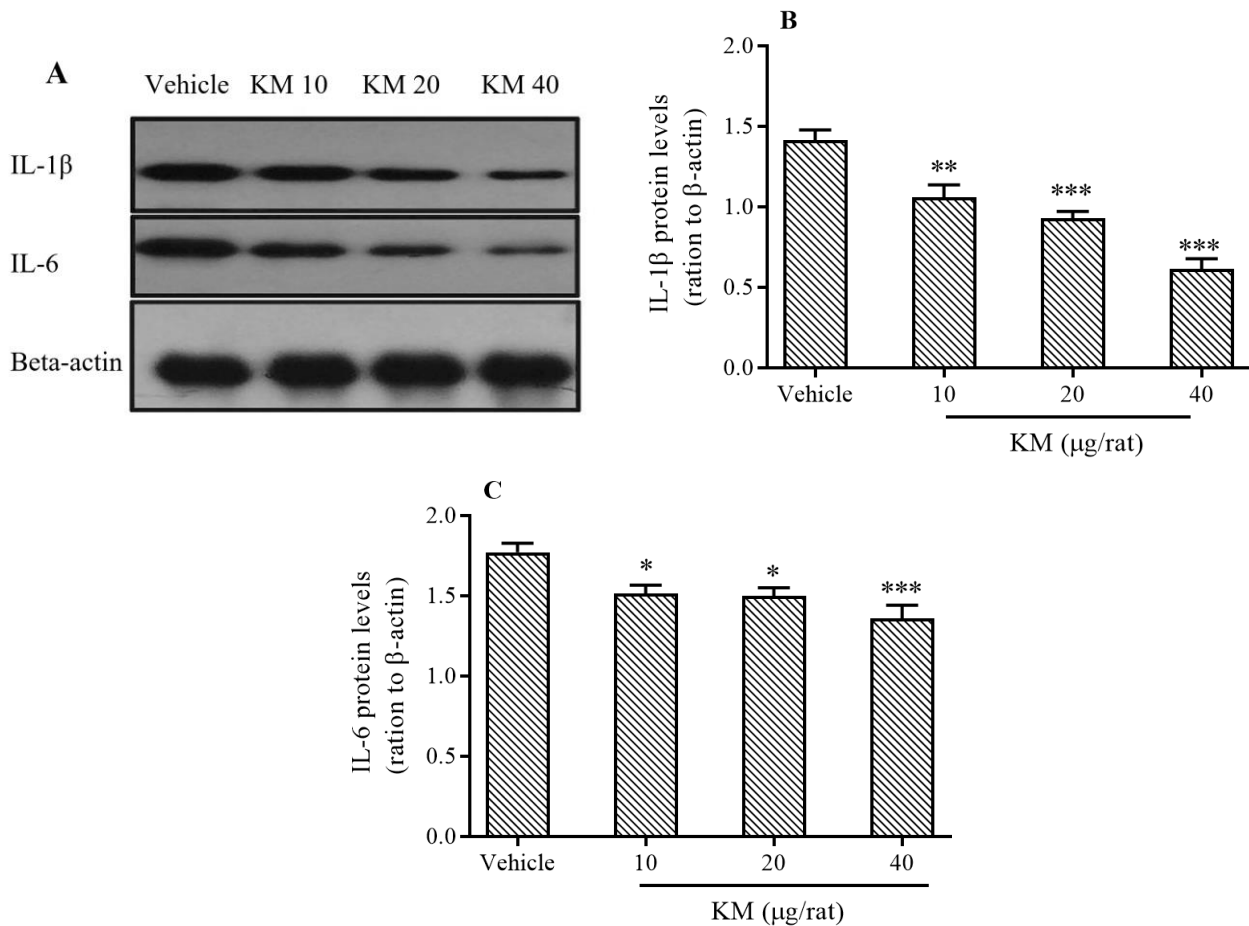


**Fig. 7.** KM did not affect (A) the number of grids that rats crossed and (B) the angle that rats slipped; n = 6. Dimethyl sulfoxide was used as the vehicle. KM, Kaempferol.



**Fig. 8.** Assessment of KM effect on the expression of p-CASP6, a-CASP6, and TNF- $\alpha$ , 60 min following formalin administration. (A) Results of western blotting analysis of p-CASP6, a-CASP6, and TNF- $\alpha$  in the spinal cord; (B-D) their expression levels; n = 6. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  show significant differences vs the vehicle group (dimethyl sulfoxide). The blots were run using a similar trial status. a-CASP6, caspase 6 active type; KM, kaempferol; p-CASP6, pro-caspase 6; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .





**Fig. 9.** Evaluation of KM effect on the expression of IL-1 $\beta$  and IL-6 60 min following injecting formalin. (A) Western blots of IL-1 $\beta$  and IL-6 in the spinal cord; (B and C) their expression levels. The blots were run using similar experimental conditions. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  significantly differ from the vehicle group (dimethyl sulfoxide). Kaempferol (KM). IL-1 $\beta$ , Interleukin 1  $\beta$ ; KM, kaempferol.

### **Role of the CASP6/TNF- $\alpha$ pathway, IL-1 $\beta$ , and IL-6 in the spinal cord**

Based on FP test results, KM led to a substantial ( $P < 0.05$ ) down-regulation of the inflammatory cytokines, such as  $\alpha$ -CASP6, p-CASP6, IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 proteins in the spinal cord that was assessed 60 min following administration. Also, 40  $\mu\text{g}/\text{rat}$  of KM reduced the expression level of mentioned proteins by 72%, 67%, 79%, 82%, and 77 %, in the respective order than the control group (Figs. 8 and 9).

## **DISCUSSION**

The present study showed that IT and/or ICV microinjection of KM could induce antinociceptive effects. It seems that the modulation of the opioidergic and GABAergic systems could be involved in this antinociceptive activity. Furthermore, KM reduced the CASP6, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6

expressions in the spinal cord. Because pain is a multifaceted phenomenon, nociceptive methods with various mechanisms are needed for evaluating the antinociceptive effects exactly (34). Based on our results, the IT injection of KM showed more effectiveness compared with the ICV injection in both TF and FP tests. Therefore, the antinociceptive effects of KM following the IT injection can be primarily owing to the effects on the spinal cord.

In the previous investigations, the IT or ICV administration of drugs in the same conditions showed different results (35-37). For example, in the study by Bustamante *et al.*, it has been reported that IT but not ICV administration of aspirin, indomethacin, and ketoprofen led to a dose-dependent depression of the C-fiber reflex. However, saline was not effective (38). Thus, in the present study, we decided to use both routes of administration. Moreover, in the study by Hara *et al.* the IT or ICV

administration of luteolin (an important flavonoid) showed different results regarding neuropathic pain. As such, spinally applied luteolin attenuated mechanical as well as cold hyperalgesia; however, supraspinally used luteolin showed no antihyperalgesic impact on neuropathic pain (36).

The results of the TF test (for measuring heat-induced pain) indicated that IT and/or ICV microinjection of KM partially led to a decrease in thermal nociception. Thus, KM possibly exerts antinociceptive activities by affecting both the brain and spinal cord. Our findings confirmed the investigation by Zarei *et al.* (39), in which they proposed that ICV injection of KM has antinociceptive effects in the TF test (through an interaction with the transient receptor potential vanilloid type 1). In contrast to the present study, Osama and Abo-Salem indicated that the administration of KM (IP) using another heat-induced pain test (tail immersion test), did not reduce the pain in male mice (13). The difference between these two investigations can be related to the route of administration. Because KM did not show strong antinociceptive effects in the TF test; thus, we speculated that KM may exert its antinociceptive effects on the other types of pain, like inflammatory pain. Accordingly, we decided to use an FP test.

The FP test is an accepted model that is employed to study pain and analgesia and can produce distinctive biphasic nociceptive responses. The non-inflammatory pain can trigger the first phase (0-5 min) by directly stimulating nociceptors, whereas the second phase (10-45 min) is induced by inflammation-induced pain linked to inflammatory cytokines. Generally, centrally acting drugs prevent these two phases, while peripherally acting drugs impede merely the second phase (40). According to our findings, KM considerably inhibited the second phase, whereas KM only at a 40 µg/rat dose exhibited analgesic activity in the first phase of the FP test. The central antinociceptive properties of KM can be proved by a reduction in the first phase. The marked activity of KM in phase 2 showed the possible important role of mediators of inflammation in the peripheral nerves or spinal cord. The findings of the FP test established the

antinociceptive effects of KM. KM (40 µg/rat) showed more effectiveness compared with Dic in the FP test (phase 1; IT and ICV). Consistent with these findings, the previous investigation showed that KM (100 mg/kg) administrated IP could attenuate licking and biting (in two phases of the FP test) in diabetic mice and reduced both phases of the formalin test (13).

According to the results of the rotarod test, KM did not cause any obvious skeletal muscle relaxation or tranquilizer activities affecting the CNS. Consequently, the reactions detected in the TF and FP tests were not caused by motor impairment or sedation; however, they revealed real antinociceptive properties. Notably, there were no significant changes in rats receiving KM; thus, the safety of KM is highlighted.

The opioid and GABA systems are the most important neurotransmitter systems associated with pain. According to Jinsmaa *et al.* findings, the µ<sub>2</sub> and δ opioid receptors play a key role in the spinal mechanism, while the µ<sub>1</sub> or µ<sub>2</sub> opioid receptors might moderate mostly supraspinal analgesic potency in the FP test (41). For highlighting the probable principal antinociceptive mechanisms of KM, the impacts of NLX, an antagonist of opioid receptors, were studied on the KM effects. According to the FP test findings, NLX entirely disallowed the antinociceptive impacts of KM on the phase 1 responses, while it partly prohibited the phase 2 responses.

Kappa-opioid receptors can mostly be found in the external superficial laminae of the dorsal horn in the lumbar spinal column. Spinal administration of κ-opioid receptor agonists is capable of producing significant antinociception (42). We found that IT administration of KM significantly reduced the pain score. Additionally, KM activity in phase 2 was not efficiently inhibited by µ- and δ-opioid receptor antagonists of Nax and Ntd; nonetheless, the κ-opioid receptor antagonist, Nbt, fully diminished the antinociceptive activity.

Our results also revealed an antinociceptive response to KM that was reduced after Bic (a GABA<sub>A</sub> competitive antagonist pretreatment). Meanwhile, neither Sac (a competitive antagonist of GABA<sub>B</sub> receptors) nor TPMPA (a competitive antagonist of GABA<sub>C</sub> receptors)

reversed the KM antinociceptive activity, which indicated that GABA<sub>A</sub> receptors affect KM antinociceptive activity. The association between flavonoids and GABA<sub>A</sub> receptors was found in the 1980s, indicating the potential of flavonoids to affect GABA<sub>A</sub> receptors as positive allosteric agents. GABA performs its action *via* ionotropic GABA<sub>A</sub>, metabotropic GABA<sub>B</sub>, and a form of ligand-gated chloride channels, GABA<sub>C</sub> receptors. Seemingly, GABA<sub>A</sub> receptors are the most significant and common inhibitory receptors of the CNS. GABA regulates pain sensation in the dorsal horn by GABA<sub>A</sub> receptors distributed in primary afferent terminals as well as dorsal horn nerve cells, such as spinal interneuron in the laminae I-III. GABA<sub>A</sub> receptors can be found in the dorsal root ganglion neurons as the primary afferents toward the CNS as well as interneurons in the spinal cord dorsal horn, which are crucial at the gate of afferent sensory signals toward the spinal cord, at which these receptors are capable of acting both from pre- and postsynaptic locations. In the presynaptic location, inhibiting the GABA<sub>A</sub> receptor of afferent sensory inputs can block somatosensory signaling toward the CNS, whereas GABA<sub>A</sub> receptor postsynaptic inhibition has shown to be associated with the regulation of the nociceptive threshold, particularly in tissue trauma and inflammation (43). Earlier studies reported the up-regulation of GABA concentration and synthesizing enzymes in tissues affected by inflammation. Studies have shown that in the pain caused by formalin, nociceptive behavior was inhibited by Bic pretreatment, indicating the involvement of GABA<sub>A</sub> receptors in the antinociceptive reaction against inflammatory pain (44). Besides the opioid- and GABA receptor-mediated effects, other mechanisms are probably involved in phase 2 reactions. It was assumed that the inflammatory cytokines of the paw and spinal cord can be decreased by KM and by designing further testing, the proposed hypothesis was validated.

According to what we discussed above, the KM anti-inflammatory activity is recommended. It is well known that injecting formalin leads to an inflammatory response causing swollen paws. Cell damage triggers the

formalin-related acute inflammation, provoking endogenous mediators generation, and consequently leading to releasing several inflammatory mediators in the paw (28). Lately, glial cells within the spinal cord make and keep sensitizing against pain due to inflammation *via* the release of the strong neuromodulators, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (45). Additionally, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 are linked to the sensitization against inflammation-related pain. According to Berta *et al.* findings, CASP6 expression is done particularly in C-fiber terminals in the superficial dorsal horn in the spinal cord and intraplantar injection of formalin caused CASP6 activation in the dorsal horn. An increase in the CASP6 level is capable of activating microglial TNF- $\alpha$  release and regulating synaptic plasticity and inflammation-caused pain (46). Moreover, a previous investigation has shown that during a late phase of the formalin test, the caspase signaling pathway causes pain in small fiber peripheral neuropathies, and these pathways are activated by inflammatory/immune mediators. Therefore, such pathways can be potential targets for new pharmacological drugs to treat inflammation and neuropathic pain (47).

Gruber *et al.* reported TNF- $\alpha$  is essential to induce spinal inflammatory pain, as well as long-term potentiation (48). Besides following a directly modulated synaptic transmission, TNF- $\alpha$  can stimulate glial cells for releasing pro-inflammatory mediators, like IL-1 $\beta$  and IL-6 enhancing synaptic transmission (49). Interestingly, according to the present results, CASP6 and TNF- $\alpha$  levels were decreased in the spinal cord after the KM administration. It seems that the antinociceptive effects of KM are related to the attenuation of CASP6/TNF- $\alpha$  levels in the spinal pathways. Ultimately, our findings revealed that in the FP test, KM considerably changed the IL-1 $\beta$  and IL-6 concentrations in the spinal cord. Accordingly, inflammatory pain activates glial cells and releases inflammatory mediators, like IL-1 $\beta$  and IL-6 in the PNS or CNS that might be involved in driving, keeping, and exasperating inflammation-associated pain. We suggest KM as an anti-inflammatory pain drug influences the CASP6/TNF- $\alpha$  pathway, specifically leading

to a reduction in inflammatory cytokine amounts, including IL-1 $\beta$  and IL-6 in the spinal cord.

## CONCLUSION

Finally, according to the TF and FP tests, KM (particularly in IT injection) has antinociceptive effects. Opioid and GABA receptors, as well as inflammatory cytokines of CASP6, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the spinal cord, are associated with the antinociceptive effects of KM. KM can be regarded as an appropriate new therapeutic option to treat inflammatory pain. Further investigations are required for validating the current data and exploring accurate mechanisms behind such significant antinociceptive activities of KM.

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## Conflict of interest statement

The authors declared no conflict of interest in this study.

## Authors' contribution

M. Bananej and R. Hajikhani conceived the experiments. S. Jabbari, M. Zarei, and A. Komaki conducted the experiments and data analysis and wrote the manuscript. The manuscript was reviewed by all authors.

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