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Original Article

Possible Involvement of Serotonergic Mechanism(s) in the Antinociceptive Effects of Kaempferol

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Abstract

Background and Objective: A flavonoid kaempferol (KM) exerts an anti-inflammatory effect and is reportedly capable of preventing metabolic diseases. Nonetheless, a limited number of studies have been carried out on the antinociceptive effects of kaempferol. The present study aimed to investigate the involvement of serotonin receptors in the antinociceptive-like activity of KM in male Wistar rats using the tail-flick test.

Materials and Methods: The compounds (i.e., KM, morphine, and diclofenac) were intracerebroventricularly administered to rats for the examination of central effects on the thermal pain using the tail-flick test. For the evaluation of the involvement of serotonin receptors in the possible antinociceptive effects of kaempferol, several antagonists (i.e., tropisetron, ketanserin, GR113808, WAY 100635, and penbutolol) were used. Additionally, locomotor activity and motor responses were investigated by the rotarod test after KM treatment.

Results: The intracerebroventricular microinjections of KM showed antinociceptive effects using the tail-flick test. The pretreatment with tropisetron as a 5-HT_3 receptor antagonist at 1 and 10 µg completely reversed the KM-related antinociception. Furthermore, ketanserin (5-HT_{2A} receptor antagonist) and GR113808 (5-HT_4 receptor antagonist) both at 10 µg reduced KM-related antinociception; however, 5-HT_{1A} receptor antagonist WAY 100635 and 5-HT_{1B} antagonist penbutolol did not decrease KM-related antinociception. All KM doses were not observed with a significant effect on locomotor activity or motor reactions.

Conclusions: The results of the current study suggested that serotonergic receptors (i.e., 5-HT_{2A}, 5-HT_3, and 5-HT_4) are effective in the KM antinociceptive activity in male rats.

Keywords: Central nervous system, Kaempferol, Pain, Serotonin receptors

Background

Pain is a major problem worldwide [1]. According to statistics, in Americans, 1 in 5 adults experiences pain each year. In addition to decreasing life quality, the pain increases the cost of general health leading to economic loss to society [2]. However, the present analgesic drugs (e.g., morphine or diclofenac [Dic]) have several side effects that are either too potent or too weak [3, 4].

Flavonoids (FV) have been known as polyphenolic compounds abundantly observed in fruits and are classified as flavones, flavanols, isoflavones, flavanones, flavonols, and anthocyanins. According to the epidemiological and pharmacological studies, the application of FV components has much effectiveness, such as anti-oxidative, anti-cancer, and anti-viral properties [5, 6]. Kaempferol (KM) (Figure 1) as an FV is available in several plants and fruits (e.g., tea leaves, endive, broccoli, strawberries, tomato, and grapes) and herbal products commonly applied in botanical medicine (e.g., *Sophora japonica* and *Moringa oleifera*). The intake of KM-containing

foods and a lower risk of many disorders, including cancer and cardiovascular diseases, are positively correlated.

The KM and some glycosides of KM show diverse pharmacological activities, such as antioxidant, anti-microbial, neuroprotective, anti-osteoporotic, anxiolytic, and anti-allergic properties [7-9]. Bian et al. demonstrated that KM inhibited many pathways associated with releasing inflammatory mediators from lipopolysaccharides-related intestinal micro-vascular endothelial cells in rats [10]. Furthermore, Qian et al., 2019 have shown that KM reduced K63-associated polyubiquitination for the inhibition
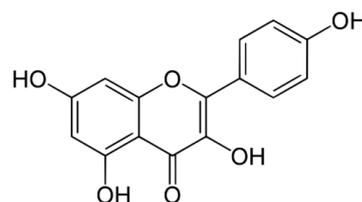

Figure 1. Chemical structure of kaempferol ($C_{15}H_{10}O_6$)

of unknown factor-kappa B and inflammatory reactions in acute pulmonary injuries in mice [11]. Nevertheless, limited reports have been issued on the antinociceptive effects of kaempferol, such as a study by Kim et al. (2015) [12-14]. None of the aforementioned studies assessed the involvement of serotonergic mechanism(s) in the antinociceptive effects of kaempferol in rats.

Objectives
The present study aimed to investigate the intracerebroventricular (ICV) antinociceptive effects of kaempferol and identify its possible causal mechanism (the involvement of selective serotonin receptors) using the tail-flick test in male rats.

Materials and Methods
Animals
Adult male Wistar rats (weighing 180-250 g) provided by Pasteur Institute, Iran, with water and food ad libitum were maintained in a 12:12 h light-dark cycle (humidity: $50 \pm 5\%$; temperature: $22 \pm 2°C$). The experiments were carried out in the light period (10:00-16:00). The examiner was blinded to the treatments, and the rats were randomly divided into different groups. There were six rats in each experimental group. All testing and animal care procedures were approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1397. 139). Moreover, the experiments were performed following the National Institutes of Health publication No. 85-23, revised in 1985.

Drugs and chemical compounds
The KM (10, 20, 40 μg/rat), dimethyl sulfoxide (DMSO), formaldehyde solution, ketamine hydrochloride, and xylazine, were all purchased from Sigma-Aldrich Corporation, USA. After dissolving KM in DMSO, it was diluted with normal saline (NS) before administration. The NS was applied for the dilution of Dic and morphine as positive controls. WAY 100635 (5-HT$_{1A}$ receptor antagonist; Sigma-Aldrich Co., USA), penbutolol (5-HT$_{1B}$ antagonist; Sigma-Aldrich Co., USA), ketanserin (5-HT$_{2A}$ receptor antagonist; Glaxo, UK), tropisetron (5-HT$_3$ receptor antagonist; Sigma-Aldrich Co., USA), and GR113808 (5-HT$_4$ receptor antagonist, ICS205930; Sandoz, France) were dissolved in DMSO. The solutions were prepared immediately prior to testing. The selected concentrations of KM and all drugs were based on previous studies and outcomes of the initial experiments of the authors of the present study [9, 15-20]. Furthermore, since NS or DMSO administration has shown the same results without

any acute or chronic toxicity, it was decided to use DMSO as a control or vehicle (Veh) group in all the experiments.

Intracerebroventricular cannulation
Firstly, the anesthetization of the rats was intraperitoneally performed using ketamine-xylazine (80 and 10 mg/kg, respectively). Then, a stainless-steel cannula (21-gauge, 12 mm) was implanted in the right lateral ventricle for ICV injection. According to the Paxinos and Watson rat brain atlas [21, 22], the stereotaxic coordinates were 1.5 mm right lateral, 0.8 mm posterior, and 4.0 mm ventral toward the bregma. Following securing the guide cannula by dental cement, it was anchored by stainless steel screws (fixed to the skull) and then sealed using stainless steel wire for the inhibition of occlusion. After 7 days of recovery, the rats were separately housed 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 PE-20 catheter, added to the guide cannula, and extended 1 mm over the guide cannula tip. Within 20 min, 10 μL of 100% DMSO or KM (10, 20, and 40 μg/rat) were administered.

Tail-flick test
The antinociceptive reaction to the thermal stimulus was measured using the tail-flick test (PANLAB 7160, Spain). The rats were restrictively held with the tail on a slot (adjustable width) with a groove to ensure accurate placement in the tail-flick apparatus to apply radiant heat to the dorsal surface of the rear set for forcing the rat to flick its tail in 2-4 sec as the tail-flick latency baseline. The gap between the start of heat exposure and tail withdrawal was calculated to determine the tail-flick latency that 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 sec for minimizing tissue injury [23, 24].

Treatment protocol
The administration of 1 and 10 μg/rat doses of the 5-HT receptor antagonist was carried out 5 min before KM ICV injection. The latency time was determined at 30, 60, 90, and 120 min at the end of the second injection. For the first experiment, the rats were assigned to the Veh, KM (10, 20, and 40 μg/rat), morphine (1 μg/rat), and Dic (1 μg/rat) groups. In the second experiment, the investigation of the 5-HT$_1$ receptor in the antinociceptive effects of kaempferol, the rodents were scrutinized to the Veh, Veh+KM,

WAY100635 (1 and 10 μg/rat), and penbutolol (1 and 10 μg/rat) groups. In the third experiment, the investigation of the 5-HT$_2$ receptor in the antinociceptive effects of kaempferol, the rodents were scrutinized to the Veh, Veh+KM, and ketanserin (1 and 10 μg/rat) groups. In the fourth experiment, the investigation of the 5-HT$_3$ receptor in the antinociceptive effects of kaempferol, the rodents were scrutinized to the Veh, Veh+KM, and tropisetron (1 and 10 μg/rat) groups. In the final experiment, the investigation of the 5-HT$_4$ receptor in the antinociceptive effects of kaempferol, the rodents were scrutinized to the Veh, Veh+KM, and GR113808 (1 and 10 μg/rat) groups [9, 25, 26]. In each group, six rats were tested.

Rotarod experiment
According to the literature [27, 28], a model 47700 accelerating rotarod (Ugo Basile, Italy) was used to examine the effect of KM on motor performance. The time to falling was calculated in seconds by placing the normal rats on a rotating drum with increasing speed (from 4 to 40 rpm in 5 min), which forced them to move onward to avoid falling. The animals' baseline responses were determined on the experiment day and the impacts of ICV and intrathecal injection of DMSO and KM on motor performance were repetitively studied for 120 min after the injections.

Safety assessment: Acute and chronic toxicity of KM
Acute and chronic toxicity testing was carried out for the assessment of KM safety. Abnormal behaviors, anomalies in terms of food intake, body weight, activities, feces, hair, and gross anatomy following Veh or KM ICV administration (10, 20, or 40 mg/kg) were studied in the first 3 days and after 14 days [29].

Data analysis
All data were expressed as mean±standard error of mean. Data analysis was carried out using SPSS software (version 16.0) and one-way or two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni post hoc test (multiple-comparison test). In each statistical comparison, a p-value of less than 0.05 was considered statistically significant.

Results
Time courses of the tail-flick response against KM ICV injection
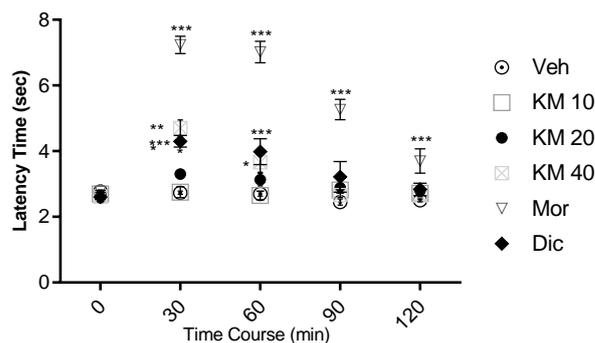According to the results of Figure 2, two-way repeated measures ANOVA showed a time effect

Figure 2. Time course regarding alterations in the tail-flick reaction against intracerebroventricularly administered kaempferol (KM) and dimethyl sulfoxide (vehicle [Veh]/control); groups of rats injected with KM (10, 20 or 40 μg/rat), morphine (Mor), diclofenac (Dic), or control/Veh (5 μl); the tail-flick responses determined at 0, 30, 60, 90, and 120 min; all data expressed as mean± standard error of mean; * P<0.05 and *** P<0.001 vs. the Veh group (n=6)

(F [4, 20]=52.78; P<0.001), group effect (F [5, 25]=117.7; P<0.001), and time × group interaction effect (F [20, 100]=14.58; P<0.001). Moreover, the obtained data demonstrated that KM (40 μg/rat) showed significant antinociceptive activities at 30 and 60 min, compared to the Veh group (P<0.001 and P<0.05, respectively). Furthermore, the microinjection of either morphine or Dic completely showed the antinociceptive effects at 30 and 60 min (P<0.001).

Impacts of 5-HT$_1$ receptor antagonists on KM-related antinociceptive effects
According to Figure 3A, two-way repeated measures ANOVA indicated a time effect (F [4, 20]=32.62; P<0.001), group effect (F [3, 15]=12.12; P<0.001), and time × group interaction effect (F [12, 60]=5.391; P<0.001). Moreover, 5-HT$_{1A}$ receptor antagonist WAY 100635 at the employed doses (1 and 10 μg/rat) did not alter antinociceptive effects induced by KM. According to Figure 3A, two-way repeated measures ANOVA showed a time effect (F [4, 20]=18.31; P<0.001), group effect (F [3, 15]=10.55; P<0.001), and time × group interaction effect (F [12, 60]=4.585; P<0.001). In a similar range of dosage, the 5-HT$_{1B}$ antagonist penbutolol also caused no significant alteration in the antinociceptive effects of KM.

Impacts of 5-HT$_2$ receptor antagonists on KM-related antinociceptive effects
According to Figure 4, two-way repeated measures ANOVA demonstrated a time effect (F [4, 20]=26.05; P<0.001), group effect (F [3, 15]=8.817; P<0.001), and time × group interaction effect (F [12, 60]=4.187; P<0.001). Moreover, 5-HT$_{2A}$ receptor antagonist ketanserin at the high dose
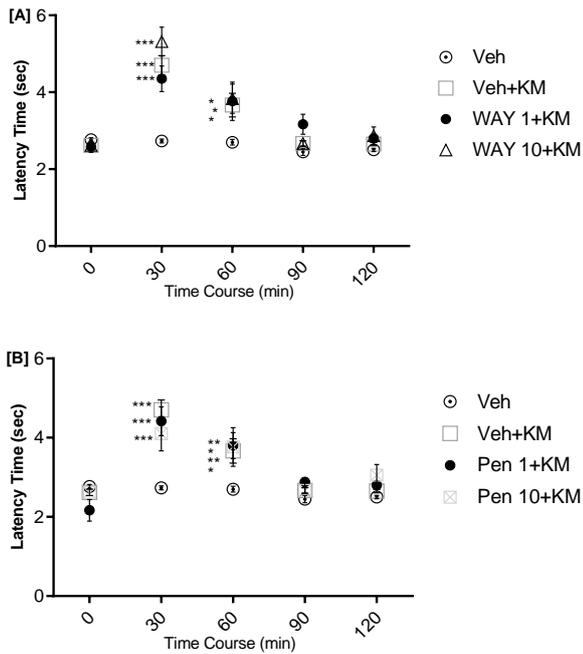
Figure 3. Role of 5-HT$_1$ receptor antagonists in the antinociceptive effects of kaempferol (KM) with dose of 40 μg/rat after intracerebroventricular injection in tail-flick test; vehicle (Veh), WAY 100635 (WAY; 1 and 10 μg/rat [A]), and penbutolol (Pen; 1 and 10 μg/rat [B]): * P<0.05, ** P<0.01, and *** P<0.001 vs. the Veh group (n=6)

(10 μg/rat) significantly altered antinociceptive effects induced by KM (P<0.01).

Impacts of 5-HT$_3$ receptor antagonists on KM-related antinociceptive effects

According to Figure 5, two-way repeated measures ANOVA showed a time effect (F [4, 20]=15.90; P<0.001), group effect (F [3, 15]=5.801; P<0.001), and time × group interaction effect (F [12, 60]=5.428; P=0.007). In addition, the KM antinociceptive activity was completely blocked during the experimental procedure following the pretreatment with tropisetron (P<0.001).
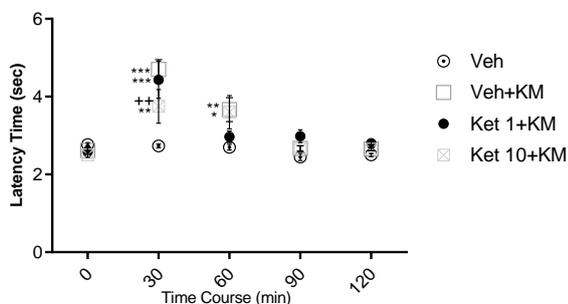

Figure 4. Role of 5-HT$_2$ receptor antagonists in the antinociceptive effects of kaempferol (KM) with dose of 40 μg/rat after intracerebroventricular injection in tail-flick test; vehicle (Veh), ketanserin (Ket; 1 and 10 μg/rat): * P<0.05, ** P<0.01, and *** P<0.001 vs. the Veh group; $^{++}$P<0.01 vs. (Veh-KM)-treated group (n=6)
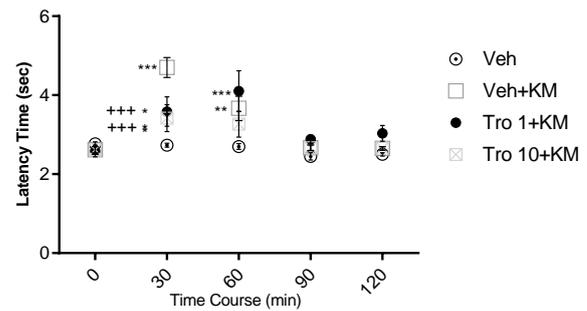

Figure 5. Role of 5-HT$_3$ receptor antagonists in the antinociceptive effects of kaempferol (KM) with dose of 40 μg/rat after intracerebroventricular injection in tail-flick test; vehicle (Veh), tropisetron (Tro; 1 and 10 μg/rat): * P<0.05, ** P<0.01, and *** P<0.001 vs. the Veh group; $^{+++}$P<0.001 vs. (Veh-KM)-treated group (n=6)

Impacts of 5-HT$_4$ receptor antagonists on KM-related antinociceptive effects

According to Figure 6, two-way repeated measures ANOVA indicated a time effect (F [4, 20]=33.19; P<0.001), group effect (F [3, 15]=11.94; P<0.001), and time × group interaction effect (F [12, 60]=5.402; P=0.007). However, the GR113808A highest dosage (10 μg/rat) caused a slight reduction in the KM antinociceptive activity (P<0.05).

Effect of kaempferol on locomotor function and motor reactions

The KM impact on locomotor function and motor reactions was explored to eliminate the probability that the KM-related antinociceptive activity is less vital compared to its sedative or muscle-relaxant properties. The dosage at which KM exerted intense antinociceptive activities did not significantly (P>0.05) affect the locomotor function or motor reactions in comparison to that of the control group (Figure 7).

Safety assessment: Acute and chronic toxicity of KM
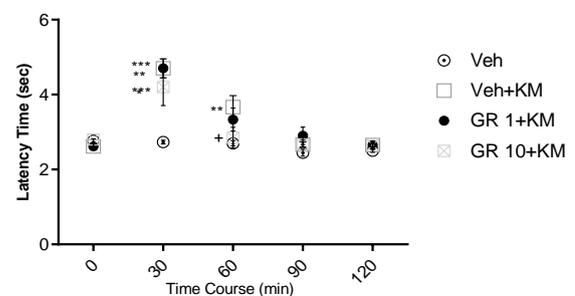
The ICV administration of KM did not lead to any

Figure 6. Role of 5-HT$_4$ receptor antagonists in the antinociceptive effects of kaempferol (KM) with dose of 40 μg/rat after intracerebroventricular injection in the tail-flick test; vehicle (Veh), GR113808A (GR; 1 and 10 μg/rat): ** P<0.01 and *** P<0.001 vs. the Veh group; $^{+}$P<0.05 vs. (Veh-KM)-treated group (n=6)
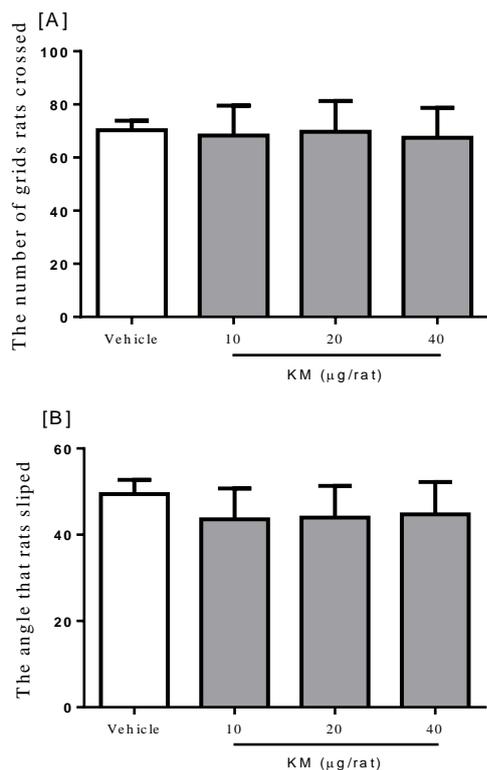
Figure 7. Role of kaempferol (KM) in locomotor activity and motor responses (n=6)

abnormal behaviors or anomalies in food intake, body weight, hair, activity, feces, and gross anatomy among the rats. There were no significant effects within the first 3 days or after 14 days, indicating no acute or chronic toxicity due to KM (data not shown).

Discussion

The most important finding of the present study was a reduction in the KM antinociceptive activity caused by the ICV injection of $5-HT_3$, $5-HT_2$, and $5-HT_4$, but not $5-HT_1$ receptor antagonists. Pain is not a unitary phenomenon; therefore, nociceptive methods with various strategies are required for the exact evaluation of the antinociceptive activity [30].

Wei-Han et al in 2019 showed that KM can attenuate neuroinflammation through passing the blood-brain barrier in the brain of rats [31]. The results obtained from the tail-flick test showed that KM ICV microinjection partially led to a decrease in thermal nociception. As a result, KM possibly exerts its antinociceptive activity affecting the brain. The results of the current study confirmed the findings of a previous study conducted by Zarei et al. [32], in which they proposed that the ICV injection of KM in the tail-flick test has antinociceptive effects (through the interaction with the transient receptor potential vanilloid-1). This finding is in contrast to the results of the present study [12].

It has been widely shown that the ICV injection of 5-HT receptor antagonists in rats or lesions in different sections of the brain related to the 5-HT system can cause hyperalgesia in the tail-flick test [33-35], indicating exerting a tonic inhibitory effect on nociceptive neurotransmission by 5-HT. Many receptors may be involved in the central antinociceptive effects of KM [32]. The findings regarding the $5-HT_3$ and $5-HT_2$ receptors should also be considered. The KM impact was fully reversed by tropisetron indicating the possible role of this receptor. The role of $5-HT_3$ in 5-HT antinociception has been widely reported [36, 37]. Nevertheless, the $5-HT_3$ ICV administration is not effective [38] or facilitates [39] nociceptive reactions. Moreover, it has been announced that the 5-HT antinociceptive activity is not associated with $5-HT_3$ receptors. Tropisetron prevented KM-associated antinociception at low and high doses, demonstrating that this receptor strongly plays a role in this mechanism.

In addition, the activation of $5-HT_2$ and $5-HT_3$ receptor subtypes can induce a cellular excitation against the $5-HT_{1A}$ receptor family. $5-HT_2$ increases phospholipase activity and $5-HT_3$ is a 5-HT ligand-gated cation channel that when activated can increase potassium/sodium conductance, resulting in cell depolarization. Furthermore, there would be a similar target for their effects on pain relief [40].

According to Dupuis et al. (2017), inhibitory potentials in rat trigeminal neurons are mediated by $5-HT_2$ receptors through the activation of GABAergic/glycinergic interneurons [41]. Concerning $5-HT_3$ receptors, another pharmacological study reported the blockage of the inhibitory activity of 2-methyl serotonin affecting nociceptive projection neurons through $5-HT_3$ or $GABA_A$ receptor antagonists [42]. Therefore, $5-HT_2$ and $5-HT_3$ receptors on GABAergic neurons can be targeted for the KM antinociceptive activity by activating such neurons providing a secondary inhibitory effect on the nociceptive projection neurons.

In the present study, the injection of both ketanserin and GR113808A partly inhibited the KM antinociceptive activity. Accordingly, some KM effects are mediated by such receptors. The effects of $5-HT_1$ receptors on the modulation of nociception have been widely studied [43, 44]; however, it is required to obtain exact data on the antinociceptive activities of $5-HT_2$ areas [34]. Nonetheless, $5-HT_2$ receptors are possibly involved in the antinociceptive impacts of periaqueductal grey stimulation and stress [45].

Both the selective 5-HT1A and 5-HT1B receptor antagonists, WAY 100635 and penbutolol, had no

significant effect on antinociception induced by 5-HT, respectively. This seems to exclude a mediation by these receptors of the antinociceptive effect of KM on a thermal pain test. The potential role of 5-HT$_4$ receptors in mediating pain has not yet been perceived. However, 5-HT$_4$ receptors possibly decrease pain [46], and 5-HT$_4$ agonists cause antinociception through cholinergic strategies [47]. The results of the present study showed that KM-induced antinociception was not reversed by a 5-HT$_4$ receptor antagonist.

According to the results of the rotarod test, KM did not cause any significant skeletal muscle relaxation or sedative impacts on the central nervous system. Consequently, the behavioral reactions detected in the tail-flick test were not caused by motor dysfunction or sedation; however, they revealed real antinociceptive properties.

Conclusions

The obtained results of the current study demonstrated the complete inhibition of the KM antinociceptive properties via 5-HT$_3$ receptor antagonist, limited influence of 5-HT$_2$ and 5-HT$_4$ receptor antagonists, and no contribution of the 5-HT$_1$ receptor. Nonetheless, through the use of low doses and receptor profiles of the ligands, some receptors' contributions could be suspected and a degree of nonselectivity cannot be excluded. Therefore, the presence of novel and more selective 5-HT agonists and antagonists is essential to perceive the serotonergic strategy involved in pain management.

Authors' contributions
Maryam Bananej and Ramin Hajikhani conceived the experiments. Sajjad Jabbari performed the research. Alireza Komaki analyzed the results and conducted the experiments, and Mohammad Zarei wrote the main manuscript. All the authors reviewed the final manuscript.

Conflicts of Interest
The authors declare that there is no conflict of interest.

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