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Effects of thymoquinone on acute corneal and orofacial pains in rats: central involvement of opioid, cannabinoid, muscarinic cholinergic, and serotonin receptors

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Abstract

Thymoquinone (TQ), the bioactive compound found in black seed, possesses beneficial properties. In the present study, the effects of oral administration of TQ were investigated on acute corneal and orofacial pains in rats. To clarify the central mechanism of action, muscarinic cholinergic, cannabinergic 1 (CB_1) and 5-hydroxytryptamine receptor antagonists were delivered into the 4th ventricle of the brain after oral administration of TQ. Acute corneal and orofacial pains were induced by dropping of hypertonic saline (50.00 µL; 5.00 M) on the corneal surface and subcutaneous injection of capsaicin (1.50 µg; 20.00 µL) in the vibrissal pad, respectively. The eye wiping number and face rubbing duration were recorded as corneal and orofacial pains behavioral responses, respectively. Locomotor activity was measured using an open-field test. The TQ (5.00 mg kg^{-1}) had no effects, while it reduced pain responses at 10.00, 20.00, and 40.00 mg kg^{-1}. Intracerebroventricular injections of naloxone (an antagonist of opioid receptors), (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (an antagonist of CB_1 receptors), atropine (an antagonist of muscarinic cholinergic receptors), and ondansetron (an antagonist of 5-hydroxytryptamine 3 receptors) at a similar dose of 10.00 µg kg^{-1} inhibited corneal and orofacial pains suppression caused by 40.00 mg kg^{-1} TQ. The tested drugs did not affect locomotor activity. It is concluded that TQ caused analgesia in the acute corneal and orofacial pains. Central opioid, cholinergic muscarinic, CB_1, and 5-hydroxytryptamine 3 receptors might be involved in the anti-nociceptive effects of TQ.

Introduction

Acute pain, particularly in the head and face, serves as a critical defense mechanism against harmful stimuli, such as surgery, illness, or injury.[1] Common treatments for acute pain include the use of acetaminophen, caffeine, ketamine, gabapentin, and opioids.[2] However, these treatments often fail to provide sufficient pain relief and may cause significant side effects, including tolerance and dependency, particularly with opioid use.[2] Medicinal plants and their active substances are recommended for optimizing pain management.[3] In this regard, knowing the neural pathways of pain being affected by natural compounds can provide new insights into the optimal pain management.[4]

Thymoquinone (TQ), the effective compound of black seed, exerts potent anti-oxidant and anti-inflammatory effects and has shown beneficial effects in disease models of various body systems, including cardiovascular, respiratory, urinary, and nervous systems.[5] For example, in Alzheimer's disease model created by aluminum chloride, TQ caused improving effects by reducing the activity of oxidative system and increasing the activity of anti-oxidant system.[6] The TQ affects pain processing mechanisms. For example, TQ was found to reduce neuropathic pain by down-regulating toll-like receptor 4 in the paclitaxel-induced peripheral neuropathic pain model.[7]

Nociceptive information from facial and oral structures, such as lips, eyes, upper and lower jaws, facial muscles, and teeth, is transmitted by trigeminal nerve to the trigeminal ganglion.[8] Many brain structures, such as trigeminal nucleus, parabrachial nucleus, thalamus, and cerebral cortex, are involved in acute pain originating from eye and orofacial regions.[9] Pain processing involves complex interactions among various neurotransmitters and neuropeptides, including gamma-amino butyric acid,

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noradrenaline, cannabinoids, acetylcholine, dopamine, serotonin, and opioids.[1]

The analgesic effect of the majority of medicinal plants occurs by changing the central mechanisms of pain processing.[4] In this regard, intracerebroventricular (ICV) injection of naloxonazine (an antagonist of mu-opioid receptors) and bicuculline (an antagonist of gamma-amino butyric acid type A receptors) was found to inhibit kaempferol-induced analgesia in tail flick and formalin tests of acute pain.[10] Also, reduction of orofacial pain induced by systemic application of crocin has been reported to be unaltered by ICV injection of naloxone (NAL).[11] Considering the above findings, this study was aimed to follow up the effects of TQ on corneal and orofacial pains. Possible supra-spinal mechanisms of TQ action were monitored by ICV administration of opioid (NAL), cannabinoid (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide [AM251]), cholinergic muscarinic (atropine [ATR]), and 5-HT_3 (ondansetron [OND]) receptors antagonists.

Materials and Methods

Animals. Adult male Wistar rats weighing 230 - 270 g were used throughout the study. During a 15-day adaptation period, the rats were housed in a laboratory room with standard conditions (temperature: 22.00 ± 0.50 ˚C, humidity: 60.00 - 70.00%, and light-dark cycle: 12 hr). Food and water were freely available. Behaviors related to acute pain were recorded between 9:00 am - 1:00 pm. The methods used in this study were approved by the Veterinary Ethics Committee of Urmia University, Urmia, Iran (Ethical Code: IR-UU-AEC-3/80).

Drugs. The used drugs, including TQ, NAL, AM251, ATR, and OND, were purchased from Sigma-Aldrich, St. Louis, USA. The TQ was dissolved in 5.00% Tween.[12] NAL, ATR, and OND were dissolved and diluted in normal saline (NS). The AM251 was dissolved in 3.00% dimethyl sulfoxide.[13]

Protocol of the study. After adaptation period, a guide cannula was inserted into the 4th ventricle of the brain. On the 10th, 14th, and 18th days after cannulation, following oral (PO) administration and ICV injection, corneal pain, orofacial pain, and locomotor activity were assessed, respectively. Blinded examiners were used for recording of animal behavior.

Animal grouping. Seventy-eight rats were used based on the following grouping: In the group 1 (vehicle + NS group) ICV injection of NS was done after PO administration of TQ vehicle (2.00 mL kg^{-1}). Groups 2, 3, 4, and 5 (TQ 5.00 + NS, TQ 10.00 + NS, TQ 20.00 + NS, and TQ 40.00 + NS groups, respectively) were treated with PO administration of 5.00, 10.00, 20.00, and 40.00 mg kg^{-1} TQ, respectively, followed by ICV injection of NS (2.00 µL). Group 6 (vehicle + NAL 10.00 group) was PO administered by TQ vehicle followed by ICV injection of 10.00 µg kg^{-1} NAL. Group 7 (TQ 40.00 + NAL 10.00 group) was PO treated with 40.00 mg kg^{-1} TQ before ICV injection of 10.00 µg kg^{-1} NAL. Group 8 (vehicle + AM251 10.00 group) received TQ vehicle before central injection of 10.00 µg kg^{-1} AM251. Group 9 (TQ 40.00 + AM251 10.00 group) received 40.00 mg kg^{-1} TQ followed by central administration of 10.00 µg kg^{-1} AM251. Group 10 (vehicle + ATR 10.00 group) was treated with ICV injection of 10.00 µg kg^{-1} ATR after TQ vehicle use. Group 11 (TQ 40.00 + ATR 10.00 group) received ICV injection of 10.00 µg kg^{-1} ATR after 40.00 mg kg^{-1} TQ. Group 12 (vehicle + OND 10.00 group) received ICV injection of 10.00 µg kg^{-1} OND after TQ vehicle application. Group 13 (TQ 40.00 + OND 10.00 group) received ICV injection of 10.00 µg kg^{-1} OND after TQ (40.00 mg kg^{-1}). In this study, TQ was PO administered approximately 50 min before ICV injection of the antagonists. This treatment method has also been used in our previous study, and the reason for this is to allow sufficient time for the substance to be absorbed through the digestive tract and reach body systems, including the brain.[12] The drug doses used in the present study were similar to previous investigations.[12-15]

Brain ventricle cannulation. Each rat was anesthetized with intra-peritoneal injection of 80.00 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 10.00 mg kg^{-1}xylazine (Alfasan;). The 4th ventricle of the brain was stereotaxically (Stoelting Stereotaxic Apparatus, Wood Dale, USA) cannulated by a 23-gauge, 13.00-mm stainless-steel guide cannula according to the following coordinates: -11.20 mm to the bregma, 0.00 mm lateral to the midline, and 7.80 mm below the surface of the brain.[16] A 10-day recovery period was considered.

Administration of thymoquinone. The PO administration of TQ (2.00 mL kg^{-1}) was done by gavage.[12] The ICV injections (2.00 µL) of the above-mentioned antagonists were performed using a 5.00 µL Hamilton syringe (Innovative Labor Systeme GmbH, Stützerbach, Germany). Central injection was performed over a period of 30 sec. The TQ was PO administered 60 min, and NAL, AM251, ATR, and OND were ICV injected 10 min before pain induction.

Corneal pain induction. Corneal pain was induced according to the previous studies.[17] Briefly, rats were placed on 30.00 × 30.00 cm wooden tables. After a 15 min adaptation period, one drop (40.00 µL) of 5.00 M NaCl solution was applied locally on the corneal surface using a fine dropper and immediately the number of eye wipes performed with ipsilateral forepaw was counted for a period of 1 min. Thereafter, the eye was washed by local corneal surface application of distilled water. The control group received a drop of distilled water on the surface of the cornea.

Orofacial pain induction. For induction of orofacial pain, each rat was placed in a plexiglass observation chamber (30.00 × 30.00 × 30.00 cm) with a mirror mounted

at 45° beneath the floor to allow an unobstructed view of the orofacial region. Following a 30-min adaptation period, capsaicin (Sigma-Aldrich) solution (1.50 µg; 20.00 µL) was subcutaneously injected into the left vibrissal pad using a 27-gauge injection needle. The face rubbing duration as a pain measure was recorded over a period of 20 min.[18]

Locomotor activity. An electronic apparatus (Borj Sanat, Tehran, Iran) was used to determine locomotor activity. The animals were gently placed in the activity box. The animal's movement caused the beam to break, and the number of beam breaks was monitored.

Cannula placement verification. At the end of the experiment (day 19), 2.00 µL of Methylene Blue was injected into the 4th ventricle. After euthanizing with high-dose ether, the brains were removed and placed in a formalin solution (10.00%). Three days later, transverse sections of the brain were provided and viewed under a loupe laboratory loop (GORDAK Instruments, Foshan, China) to observe Methylene Blue distribution in the 4th ventricle (Fig. 1) according to the atlas of Paxinos and Watson.[16]

Statistical analysis. GraphPad Prism (version 8.0; GraphPad Software Inc., San Diego, USA) was used for statistical analysis. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test. All data were expressed as mean ± standard error of the mean. Statistically significant level was set at $p < 0.05$.

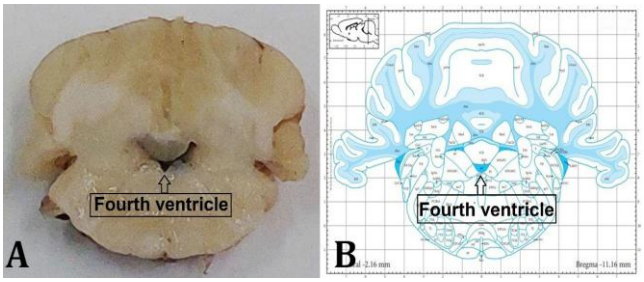

Fig. 1. A) Permanent cannula location in the 4th ventricle (arrow) of all rats included in the data analysis; **B)** Schematic illustration of transverse section of the rat brain showing the location of the 4th ventricle. Atlas plate was adopted from Paxinos and Watson.[16]

Results

Figure 1 shows cannula tip placement in the 4th ventricle. The cannula tip placement location has been shown with an arrow (Fig. 1A). Figure 1B was adopted from the atlas of Paxinos and Watson.[16]

Figure 2 shows TQ effects of on corneal (Fig. 2A) and orofacial (Fig. 2B) pains. Regarding corneal pain, ANOVA (one-way) showed significant differences ($p < 0.0001$; Fig. 2A) among groups. Subsequent Tukey's test application expressed that 10.00, 20.00, and 40.00 mg kg^{-1}, but not 5.00 mg kg^{-1} TQ decreased the number of eye wipes (Fig. 2A). One-way ANOVA revealed significant differences ($p < 0.0001$; Fig. 2B) among treated groups when orofacial pain results were analyzed. Further analysis by Tukey's test expressed that 5.00 mg kg^{-1} TQ was without effect, whereas at doses of 10.00, 20.00, and 40.00 mg kg^{-1}, it reduced face rubbing time (Fig. 2B).

Figure 3 shows central effects of NAL, AM251, ATR, and OND on the reducing effects of systemic TQ in corneal (Fig. 3A) and orofacial (Fig. 3B) pains. Considering corneal pain, one-way ANOVA revealed significant differences ($p < 0.0001$; Fig. 3A) among treated groups. Subsequent analysis with Tukey's test expressed that ICV injections of NAL, AM251, ATR, and OND at a similar dose of 10.00 µg kg^{-1} inhibited the reducing effect of 40.00 mg kg^{-1} TQ (Fig. 3A). One-way ANOVA revealed significant differences ($p < 0.0001$; Fig. 3B) among treated groups when orofacial pain results were regarded. Further analysis by Tukey's test revealed that ICV injections of NAL, AM251, ATR, and OND at a similar dose of 10.00 µg kg^{-1} inhibited the reducing effect of 40.00 mg kg^{-1} TQ (Fig. 3B).

Figure 4 shows the effects of PO administration of TQ, and ICV injections of NAL, AM251, ATR, and OND on the locomotor activity. Beam break number in vehicle-treated group was 103 ± 5.11 in a 5-min session. The PO and ICV administrations of chemicals did not significantly ($p > 0.05$; Fig. 4) influence beam break number.

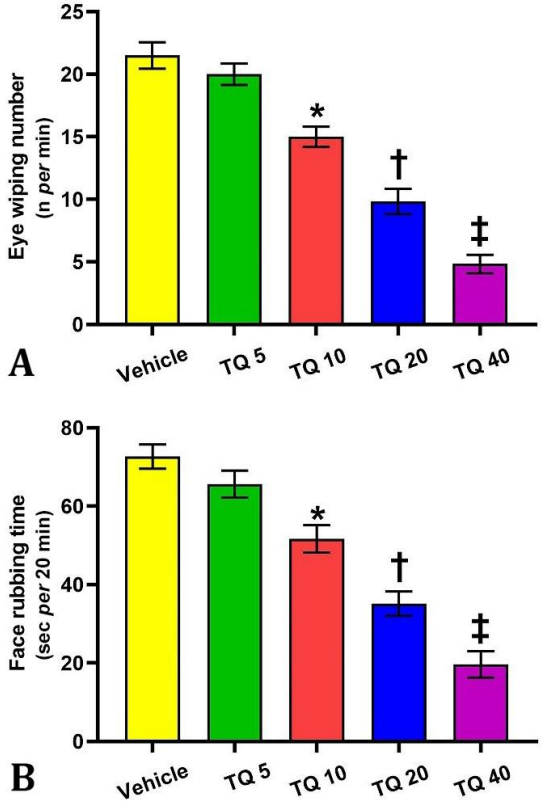

Fig. 2. Effects of thymoquinone (TQ) on **A)** eye wiping number and **B)** face rubbing time. The TQ was orally administered 60 min before corneal and orofacial pains induction. The TQ doses are in mg kg^{-1}. * $p < 0.05$, † $p < 0.01$, and ‡ $p < 0.001$ in comparison with vehicle-treated group.

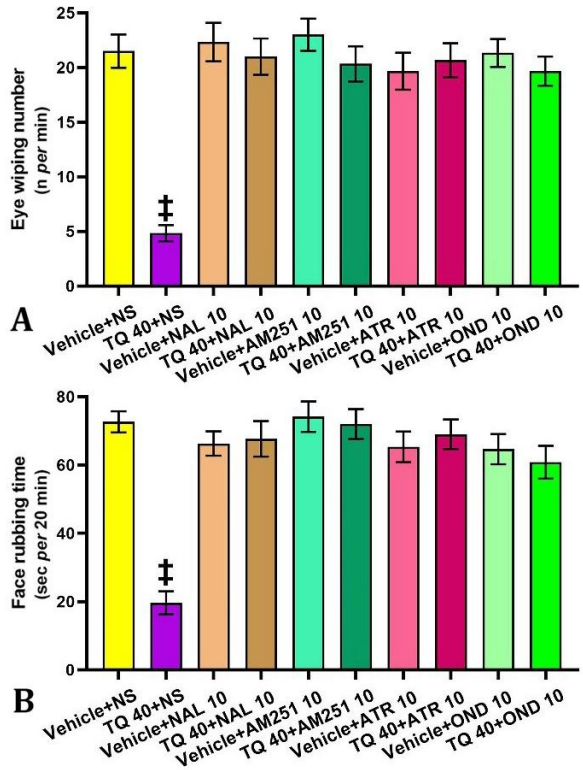

Fig. 3. Effects of intracerebroventricular injection of normal saline (NS), naloxone (NAL), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), atropine (ATR), and ondansetron (OND) alone and after vehicle and thymoquinone (TQ) on **A)** eye wiping number and **B)** face rubbing time. The TQ was orally administered 60 min, and NAL, AM251, ATR, and OND were intracerebroventricularly injected 10 min before pain induction. The TQ doses are in mg kg^{-1}, and NAL, AM251, ATR, and OND doses are in μg kg^{-1}. ‡ $p < 0.001$ in comparison with other groups.

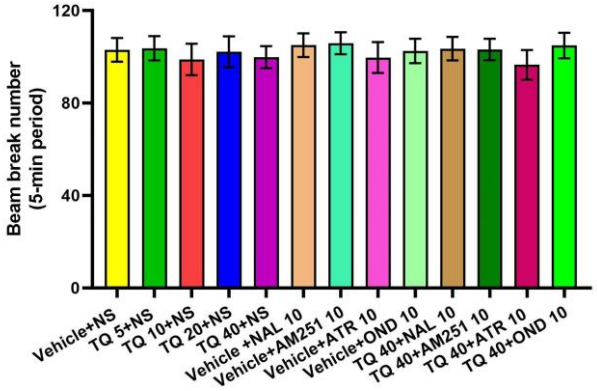

Fig. 4. Total number of beam breaks in an open-field test after administrations of vehicle, thymoquinone (TQ), normal saline (NS), naloxone (NAL), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), atropine (ATR), and ondansetron (OND) alone and after TQ. Vehicle and TQ was orally administered 60 min, and NS, NAL, AM251, ATR, and OND were intracerebroventricularly injected 10 min before locomotor activity test. The TQ doses are in mg kg^{-1}, and NAL, AM251, ATR, and OND doses are in μg kg^{-1}. There were no significant differences among treated groups.

Discussion

In the current study the number of eye wipes was 21.51 ± 1.06 in 1 min. This finding is in line with the findings of other researchers, in which the number of eye wipes was reported at about 18 *per* min following corneal stimulation by hypertonic saline (5.00 M).[19,20] Also, in the present study, the duration of face rubbing was 72.67 ± 3.08 sec *per* 20 min, which is consistent with other studies used capsaicin injection into the vibrissal pad as an orofacial pain model.[20-22]

The current study results showed that TQ dose-dependently reduced pain intensity in both corneal and orofacial pain models. There are no reports showing TQ effects on the eye and orofacial pains. It has been found that the neuroprotective effects of TQ in neurodegenerative conditions are due to the easy passage of TQ through the blood-brain barrier.[23] In this regard, TQ has been found to affect pain perception at the local and central locations after local, oral, and central deliveries. For example, in the formalin-induced pain model, it has been found that intra-plantar and ICV injections of TQ before intra-plantar injection of formalin reduce pain intensity at the 1st and 2nd phases.[24] Moreover, PO administration of TQ has been reported to reduce the number of abdominal wall contractions induced by intra-peritoneal injection of acetic acid.[12] Also, PO administration of TQ has reduced neuropathic and formalin pain responses along with improving the blood levels of malondialdehyde and interleukin-6.[25] The findings mentioned above indicate that TQ attenuates not only acute pain but also chronic pain.

In the current study, ICV administration of NAL inhibited the pain-reducing effect of TQ. The NAL is a competitive antagonist of opioid receptors with a high affinity to mu-opioid receptor.[26] Opioid receptors are distributed throughout pain pathways and play a crucial role in local, spinal, and supra-spinal processing of acute and chronic pains.[27,28] The NAL has been frequently used to clarify the involvement of opioid-dependent and non-dependent pain mechanisms in the function of synthetic and natural substances. For example, in the paw formalin and incision mechanical allodynia models of pain, intra-thecal administration of NAL has been found to antagonize with analgesic effects of pentazocine (an opioid receptor agonist) and neostigmine (a cholinesterase inhibitor).[29] In addition, it has been reported that NAL prevents the anti-nociceptive effects of kolaviron (*Garcinia* bioflavonoid) in acetic acid-induced writhing and formalin-induced licking models of pain.[30] There are few reports regarding the involvement of opioid receptors in the analgesic action of TQ. However, ICV injections of NAL, naloxonazine (an antagonist of mu_1-opioid receptors), nor-binaltorphimine (an antagonist of kappa opioid receptor), but not naltrindol (an antagonist of delta opioid receptors) have

been found to prevent the anti-nociceptive effect of *Nigella sativa* oil and TQ.[31]

Current study results showed that ICV administration of AM251 inhibited the anti-nociceptive effects of TQ. This suggests that the cannabinoid system's CB_1 receptor might be involved in the supra-spinal processing of acute pain. The functions of the cannabinoid system in the body are carried out through CB_1 and CB_2 receptors, and due to the widespread distribution of the CB_1 receptor in pain pathways, its involvement in the processing of acute pain has been strongly suggested.[32] In this regard, it has been found that ICV injection of AM251 inhibits the analgesic effect of TQ in a rat model of acute visceral pain.[12] Moreover, ICV injection of AM251 before anandamide (an endogenous cannabinoid) has been found to prevent the reduction in tongue amplitude induced by dental pulp stimulation in rats.[33] It is important to note that the CB_2 receptor, due to the wide distribution in the microglia and human primary sensory neurons, has been suggested to play a role in inflammatory and neuropathic pains processing.[34,35]

In the present study, ICV injection of ATR inhibited corneal and orofacial pains suppression induced by TQ. This indicates that cholinergic muscarinic may have a role in the TQ action in acute pain modulation. Cholinergic system, including muscarinic and nicotinic cholinergic receptors, acetylcholinesterase inhibitors, and acetyl-choline increase enhancers, involves in central modulates of acute and chronic pains.[36] Atropine is a muscarinic cholinergic receptors antagonist being widely used to investigate the involvement of these receptors in peripheral and central pains processing.[37] For example, it has been found that subcutaneous and ICV injections of ATR inhibit physostigmine-induced anti-nociception in acute corneal pain in rats.[38] There are no reports demonstrating the involvement of cholinergic muscarinic receptors in the analgesic effect of TQ. In other conditions, such as blood pressure regulation, in has been found that the *N. sativa* volatile oil and TQ dose-dependently lower the arterial blood pressure and heart rate in anesthetized rats, and ATR inhibits this effect.[39]

In the current study, TQ-induced anti-nociceptive effects were prevented by OND, suggesting the supra-spinal involvement of 5-hydroxytryptamine ($5\text{-}HT_3$) receptor in modulation of acute pain. It is well known that serotonin through $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$, and $5\text{-}HT_7$ receptors exerts potent role in brain modulation of acute and chronic pains.[40] It has been reported that intra-thecal administration of OND reverses behavioral changes (hyperalgesia) in a neuropathic pain induced by brachial plexus avulsion in rats.[41] In addition, pre-treatment of the raphe nucleus with OND was found to prevent intra-habenular morphine induced anti-nociception.[42] Although the interaction between TQ and serotonin receptors in central pain processing has not been reported, TQ has restored the brain levels of dopamine, acetylcholine, and serotonin in aluminum chloride- induced toxicity in rats.[43]

The results of the present study, in line with the findings mentioned above, confirm that at the brain level, TQ recruits several neurotransmitter mechanisms to exert its pain-relieving function. In this regard, it has been reported that in the acetic acid-induced visceral nociception, opioid and adrenergic receptors in the brain are involved in the anti-nociceptive function of TQ.[12] In addition, in the rat model of paw incision-induced pain, TQ reduced spontaneous and evoked pain responses by inhibition of cyclooxygenase-2 and malondialdehyde productions.[44] Moreover, in the acute pain models, including hot plate and cold plate tests, TQ administration reduced pain responses, and in the vincristine-induced neuropathic pain model, TQ attenuated pain responses along with a reduction in sciatic nerve malondialdehyde level.[25] It has been reported that TQ easily crosses the blood-brain barrier, acts as a neuromodulator in the brain, and influences neurotransmission.[23] However, to accurately investigate the central mechanisms of the effect of TQ on pain, studies with its administration into the ventricles or nuclei of the brain, protein docking, and receptor expression are necessary.

In the current study, PO administration of TQ, and ICV injections of NAL, AM251, ATR, and OND did not affect the locomotor activity of animals. This result is in line with the findings of previous research in which the ineffectiveness of TQ on motor behavior was reported.[12] When investigating the effects of a chemical on pain, side effects, such as effects on locomotor activity, should be considered; so that, the consequences of decreased or increased movement can be distinguished from analgesic action.[45]

In conclusion, the results of the present study showed that PO administration of TQ produced anti-nociceptive effects in corneal and orofacial tests of pain with no effect on locomotor activity. This effect of TQ was inhibited by ICV injections of NAL, AM251, ATR, and OND, indicating the involvement of supra-spinal opioid, CB_1, muscarinic cholinergic, and $5\text{-}HT_3$ receptors in the function of TQ. Research on the recruitment of brain neurotransmitter systems by natural products helps effectively in the optimal management of acute pain.

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Conflict of interest

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

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