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Original Article

Central possible antinociceptive mechanism of naringin

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ABSTRACT

Background and Aims: The object of this study was the investigation of the central antinociceptive effects of naringin as well as the association of stimulation of opioidergic, serotonergic, adrenergic, and cholinergic (muscarinic and nicotinic) receptors to the central analgesia of mice due to naringin.
Methods: Several intraperitoneal doses (20, 40, and 80 mg/kg) were injected into mice models and analyzed via hot-plate (integrated supraspinal response) and tail-immersion (spinal reflex) for the possible antinociceptive effects of naringin. Moreover, the involved action mechanism was investigated using 80 mg/kg naringin (i.p.) administered to the mice which were previously pre-treated with opioid antagonist naloxone (5 mg/kg, i.p.), serotonin 5-HT2A/2C receptor antagonist ketanserin (1 mg/kg, i.p.), α2-adrenoceptor antagonist yohimbine (1 mg/kg, i.p.) and muscarinic antagonist atropine (5 mg/kg, i.p.), as well as nicotinic antagonist mecamylamine (1 mg/kg, i.p.).
Results: It can be claimed that a dose-dependant antinociceptive effect of naringin was noticed for 40 and 80 mg/kg doses in tail-immersion and hot-plate tests, respectively. Furthermore, the improvement of inactivity of naringin-induced response to thermal stimuli was counteracted by mecamylamine and naloxone when tested with the tail-immersion test, and hot-plate analyses.
Conclusion: From the data, it was confirmed that naringin presents central antinociceptive effects which may be coordinated by supraspinal/spinal mediated opioidergic and nicotinic (cholinergic) inflection. Nevertheless, it is unclear how naringin organizes the interactions of the aforementioned modulatory systems. To conclude, naringin could be a possible candidate for pain relief management.

Keywords: Antinociception, opioidergic receptors, nicotinic antagonist, pain, central mechanisms, naringin

INTRODUCTION

Pain, in accordance with the definition by the International Association for the Study of Pain, can be described as an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Dziechciaż, Balicka-Adamik, & Filip, 2013). This phenomenon affects the central nervous system (CNS) and peripheral nervous system (PNS) in such a manner that sensory input is first transferred to the spinal cord via the PNS and then transmitted to higher centers in the CNS for perception and the final analysis (Mahmoudvand et al., 2020). During pain chronicity, a variety of activity-dependent and disease-related alterations might take place over the peripheral and central components of the somatosensory nociceptive pathway. Nociceptive pathways are subject to modulation from a plethora of hormonal and neurotransmitter systems, along with dopaminergic, serotonergic, adrenergic, and cholinergic pathways (Naser & Kuner, 2018).

The management of pain is very important for any clinician since both acute and chronic pains are significant health problems that influence patients' quality of life . Currently, numerous analgesic drugs are available in clinical practice; some of them present low analgesic activity leading the researchers to develop innovative compounds that effectively manage pain-

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ful situations, especially chronic pains. Therefore, researchers have directed their attention to natural substances that present encouraging analgesic or other pharmacological properties (Araújo et al., 2017).

Flavonoids, an important group of polyphenolic substances, are secondary metabolites and a source of bioactive molecules in plants. Their widespread availability along with their low toxicity has presented them as potent therapeutic candidates (Hui et al., 2017; Chen, Qi, Wang, & Li, 2016). Naringin is a flavanone glycoside comprised of naringenin, an aglycone, and neohesperidose attached to the hydroxyl group at C-7. It has been reported to have a bitter taste because of the presence of glucose moiety (Bharti, Rani, Krishnamurthy, & Arya 2014). In addition, naringin, alternatively known as 5,7,4′-trihydroxy flavanone-7-O-rhamnoglucoside, is a well-investigated flavanone glycoside of grapefruits and citrus fruits (Zeng et al., 2019). Various reports have disclosed that naringin might modulate several signaling pathways; consequently, it presents extensive pharmacological indexes, such as anti-inflammatory, anti-cancer, prokinetic, pro-osteogenic, antiresorptive, and antiadipogenic responses. Moreover, naringin can positively affect cardiovascular diseases, metabolic syndrome, oxidative stress, genetic damage, and neurological ailments (Li, Wu, Wang, & Su 2020).

Herein, the investigation of the potential central antinociceptive mechanism of naringin and the evaluation via pharmacological approaches of the role of the opioidergic, serotonergic, adrenergic, and cholinergic pathways in its analgesic effects, took place. Therefore, the current study provides further insights into the mechanism of antinociception induced by Naringin.

MATERIALS AND METHODS

Chemicals and materials

Naringin, carboxymethylcellulose, yohimbine hydrochloride, diclofenac sodium, ketanserin tartrate, mecamylamine hydrochloride, naloxone hydrochloride, and atropine sulfate were obtained from Sigma, Germany. All the other used chemicals were of analytical grade, if not otherwise stated.

Animals

In the current study, Adult Swiss Albino male mice with a weight of almost 20-30 g were utilized. A temperature at 20-25°C, 55±15% relative humidity, 12 h light/dark cycles were adjusted for the experiments. The mice were acclimated for seven days prior to the dose initiation and they had free access to food and water. Moreover, the animals fasted overnight prior to the behavioral tests in order to reduce variability in investigatory parameters such as food-naringin interaction, while the analyses were performed between 9.00-13.00 a.m. The experimental protocols were carried out with the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised in 1985). The ethical committee of Ege University approved all the experiments while the experiments performed on mice were done with extra care and concern (Approval no:2020/079).

Drugs and treatments

The animals were grouped into seventeen and each group comprised of six mice. The control group received only solvent vehicles. Diclofenac (10 mg/kg) was used as a reference drug (Moniruzzaman & Imam, 2014; Afify, Alkreathy, Ali, Alfaifi, & Khan 2017). Naringin was administered intraperitonally (i.p.) at the doses of 20, 40, and 80 mg/kg. All drugs were injected intraperitoneally. For investigating the mechanisms of action, the mice were pre-treated with 5 mg/kg muscarinic receptor antagonist atropine 15 min before, 1 mg/kg nicotinic receptor antagonist mecamylamine 20 min before, 1mg/kg serotonin 5-HT2A/2C receptor antagonist ketanserin 30 min before, 1 mg/kg α2-adrenoceptor antagonist yohimbine 30 min before, and 5 mg/kg opioid antagonist naloxone 15 min before the administration of 80 mg/kg naringin. The measurements of pain threshold were performed using hot-plate and tail-immersion tests 30 min after Naringin administration. Doses and drug administration schedules were selected based on previous reports (Ben-Azu et al., 2018).

Analgesia test procedures

Hot-plate test

Eddy's hot plate method was performed to assess the antinociceptive effect of naringin. Mice showing quick responses like jumping and withdrawal within 15s to thermal stress were selected for the hot plate test analysis. Twenty-four hours before the experimental procedure the mice selection was performed. The pain reflexes concerning a thermal stimulus were estimated using a temperature-controlled plate (Hot/Cold Plate, Ugo Basile, Italy). The mice from each group were placed on the plate at 55.0±0.5 °C. The reaction times of the nociceptive responses (fore paw licking, withdrawal of paw, or jumping) were recorded. To minimize tissue damage, a cut-off time of 20s was selected. The behaviors of the mice were recorded before as well as after treatment (Bektaş & Arslan, 2016; Arslan, Aydin, Samur, & Bektas, 2018).

Tail- immersion test

A hot water bath was used to dip the mice tails tips in order to assess the painful reaction of the mice induced by thermal stimulus at 52±0.2°C. More precisely, a specific area of the mice tail was immersed in hot water. Sudden removal of the tail from the hot water was considered as a pain response. A cut of period of 15s was observed to avert tail tissue destruction of the mice (Bektaş & Arslan, 2016; Arslan et al., 2018).

The antinociceptive activity was expressed as the percent maximum possible effect (%MPE) calculated as:

%MPE = [{(Postdrug latency) – (Predrug latency)}{(Cut off time)– (Predrug latency)}] X 100.

Statistical analysis

Data were analyzed by using GraphPad Prism 5.0 software, Inc., San Diego, CA. The data have been represented as mean±SEM (standard error of the mean). Moreover, the data was interpreted by using one-way analysis of variance accompanied by post hoc analysis with Tukey's test. A p-value less than 0.05 was considered significant.

RESULTS

Analysis of the possible mechanism of action of naringin

The i.p. administration of naringin 20 mg/kg, naringin 40 mg/kg ($p<0.05$), and naringin 80 mg/kg ($p<0.01$) produced a dose-dependent prolongation of the latency for mice to respond to pain stimulation induced by heat. However, naringin 20 mg/kg did not demonstrate any significant change in the antinociceptive response of the mice when compared with vehicle control. The injection of the naringin vehicle did not induce hyperalgesia or any anti-hyperalgesic effect. Meanwhile, treatment with the reference drug, diclofenac (10 mg/kg, i.p.) exerted a significant increase ($p<0.001$) in response latency against thermal stimulus-induced nociception compared to the control. Figure 1 depicts the MPE% values which illustrate the antinociceptive effect of naringin according to hot-plate and tail-immersion analyses, respectively.

Mechanism of action studies

It can be said that atropine (5 mg/kg, i.p.), mecamylamine (1 mg/kg, i.p.), ketanserin (1 mg/kg, i.p.), yohimbine (1 mg/kg, i.p.) and naloxone (1 mg/kg, i.p.) did not exhibit any significant effect on the pain threshold, according to the results of hot-plate and tail-immersion tests in mice.

As it is shown in Figure 2, the antinociceptive action of naringin was prevented due to the administration of naloxone (opioid receptor antagonist) in the hot-plate ($p<0.01$) and the tail-immersion ($p<0.001$) analyses.

As shown in Figure 3, it was revealed that pre-treatment with mecamylamine reversed the pain relief effects of the naringin in hot-plate ($p<0.001$) and tail-immersion ($p<0.01$) tests.

On the contrary, Figure 4 shows that the pre-treatment with atropine did not significantly alter the pain thresholds in both the tail-immersion and hot-plate tests.

As shown in Figure 5, ketanserin alone did not significantly alter the pain thresholds. Naringin (80 mg/kg) plus ketanserin showed a significant effect on pain response as compared to the control group in the hot-plate ($p<0.01$) and tail-immersion ($p<0.001$) tests. The results indicate that ketanserin did not reverse the antinociceptive effect of naringin in a significant way.

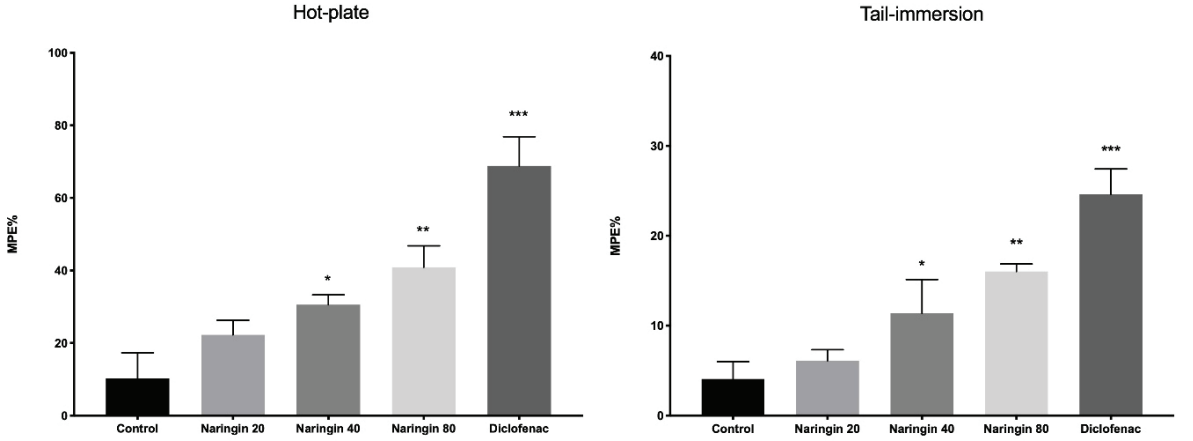

Figure 1. The induction of antinociception after naringin administration (20, 40, and 80 mg/kg) (ip) according to hot-plate and tail-immersion analyses. *p<0.05, **p<0.01, ***p<0.001 vs. Control.

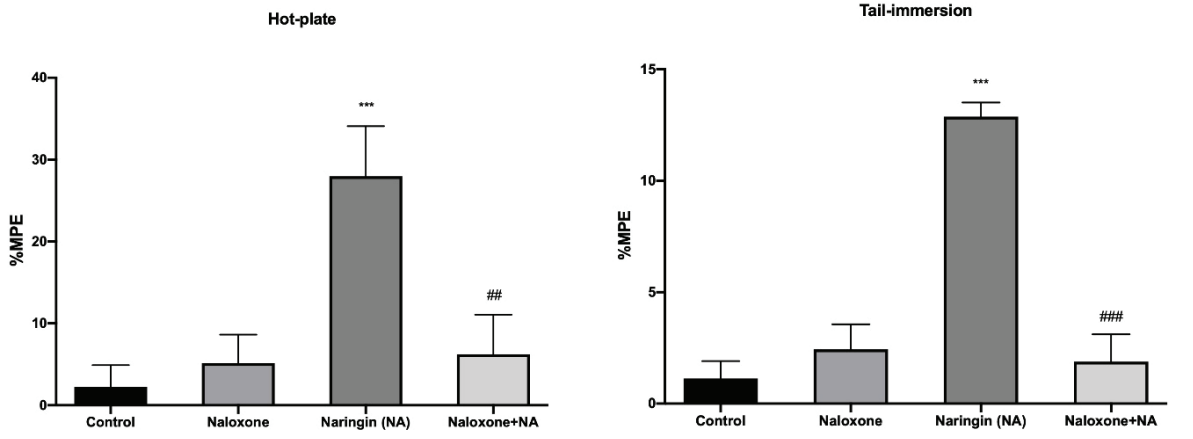

Figure 2. The reversal effect of 5 mg/kg naloxone (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. ***p<0.001 vs. Control; ##p<0.01, ###p<0.001 vs. naringin alone.

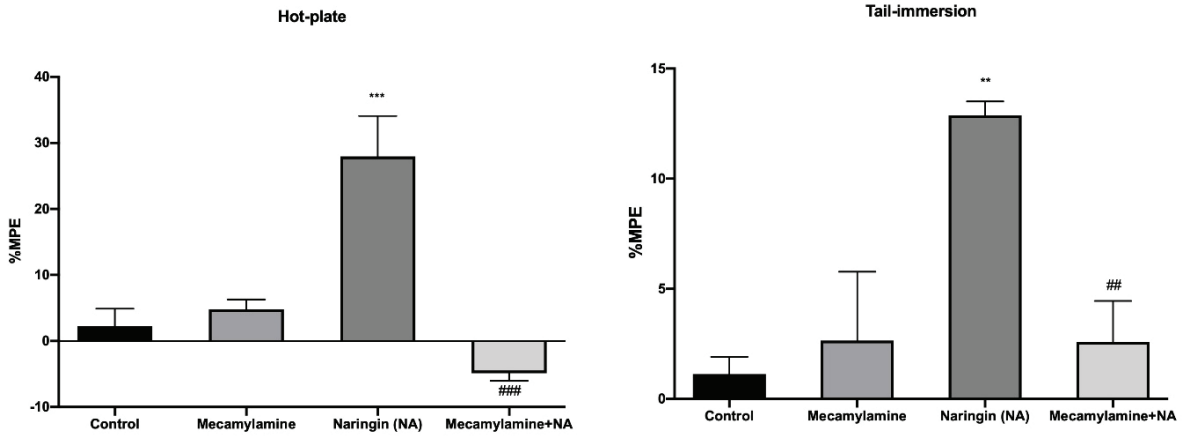

Figure 3. The reversal effect of 1mg/kg mecamylamine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control; ##p<0.01, ###p<0.001 vs. naringin alone.

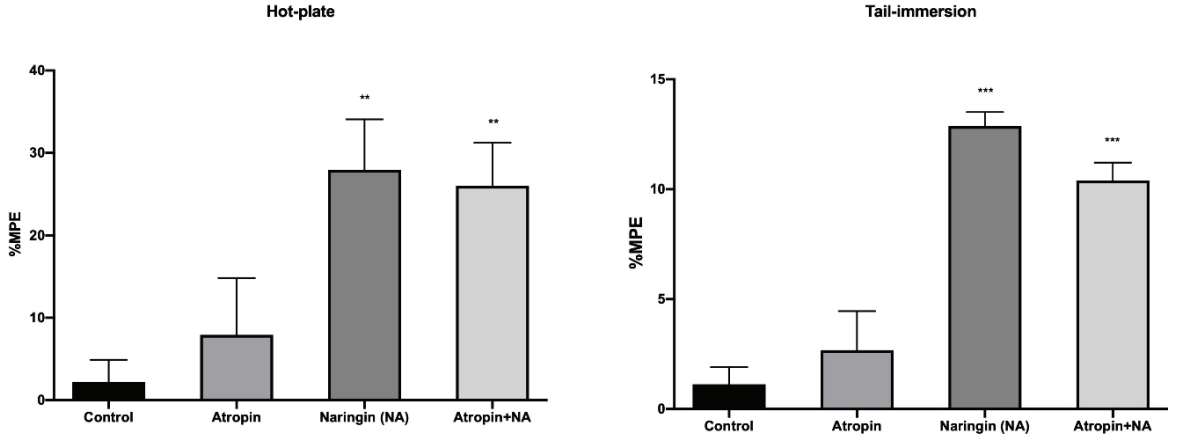

Figure 4. The reversal effect of 5 mg/kg atropine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

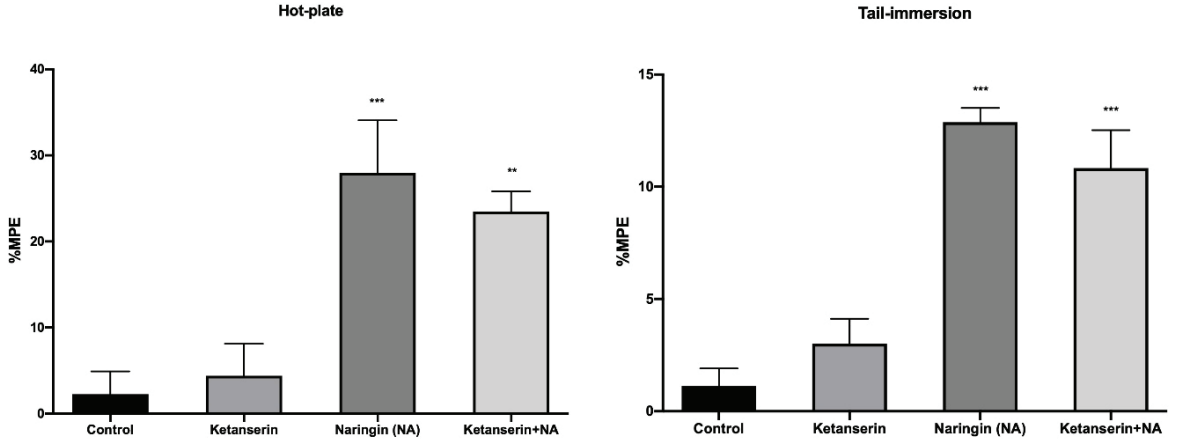

Figure 5. The reversal effect of 1 mg/kg ketanserin (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

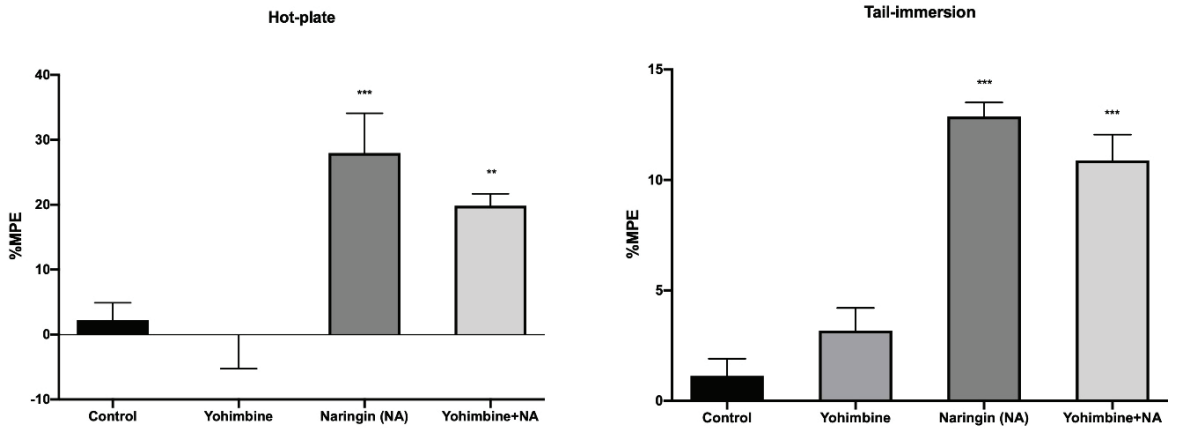

Figure 6. The reversal effect of 1 mg/kg yohimbine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

Figure 6 demonstrates that the pre-treatment with yohimbine did not show an antagonistic effect on antinociceptive activity of naringin (80 mg/kg) in the tail-immersion (p<0.01) and hot-plate (p<0.001) tests.

DISCUSSION

Herein, an investigation on the possible central antinociceptive effects of naringin through several pharmacological paths, such as hot-plate test and tail-immersion test, took place. The obtained results revealed that Naringin demonstrates statistically significant antinociceptive effects on the treated mice in comparison to control treated mice. It can be suggested that the antinociceptive effect as demonstrated by the tail-immersion and hot-plate tests, was mostly mediated by opioidergic and cholinergic systems.

In general, nociception is a reflex response of an organism due to external stimuli. Various nociceptive tests can be executed in order to examine the pharmacological effect of drugs to decrease pain. These tests can be implemented via the application of stimuli such as thermal, mechanical, or electrical. Herein, the antinociceptive potential of Naringin against the thermal stimulus provoked nociception was examined through hot plate and tail-immersion analyses; these tests are widely applied for the detection of possible analgesic properties of drugs (Xue, Wu, Wu, & Wang 2019). More specifically, the hot-plate test can be used in order to evaluate analgesics that centrally act and increase the pain threshold of animals regarding heat. It is thought to be responsive to opioids and measures the complex response to a non-inflammatory, acute nociceptive stimulus (Meshram, Kumar, Rizvi, Tripathi, & Khan, 2015). Moreover, the simple tail immerison as an endpoint of the test may be mediated as a spinal reflex. It is believed that the escape reaction is a complex occurrence liaised by the brain. Subsequently, the drug influence on the brain can be assessed by observing the escape reaction (Meshram et al., 2015). In addition, the hot-plate analysis is applied in order to assess the effects of centrally but not peripherally acting analgesics. Accordingly, the tail-flick method is useful so as to establish acute spinally mediated-nociception to thermal noxious stimuli (Chung, Li, Lin, & Tsai, 2019).

Naringin, after its oral administration, is mostly hydrolyzed to its aglycon, naringenin, by the enterobacterial enzymes, including b-rhamnosidase and a-glucosidase (Zeng et al., 2019; Li et al., 2013). Naringenin presents several pharmacological effects, such as antioxidant, anti-inflammatory, analgesic, etc. The antinociceptive properties of naringenin (66 mg/kg, i.p.) were shown with the participation of glutamatergic and opioid systems. Naringenin also reduced the nociceptive response of the formalin test (the inflammatory phase), bradykinin, and prostaglandin E2 (Dallazen et al., 2017). Only small amounts of naringin are available in plasma after oral intake and thus they might not adequately reach target tissues, such as the brain. Nonetheless, if it is given i.p., central activities of naringin have been revealed in mice (Yow et al., 2011; Nagi, Pineyro, Swayne, Tian, & Dascal 2014).

Naringin has been classified as a relatively harmless or non-toxic substance. The oral single dose of 16 g/kg of naringin has not provoked any acute toxicity when studied in the rat model (Li et al., 2013). Furthermore, according to recent research, the oral exposure of rats with a single dose of naringin (5 g/kg) did not show any mortality or unfavorable clinical effects. In addition, gross pathological signs, abnormal alteration in body weights, or toxicologically relevant modifications in serum biochemistry, hematology, urinalysis as well as other findings were not observed (Li et al., 2020).

According to the present results obtained from the tail-immersion and hot-plate tests, the pre-treatment with naloxone, an opioid receptor antagonist of μ-, κ- and δ-opioid receptors (MOR, DOR and KOR, respectively) (Araújo et al., 2017) has antagonized the antinociceptive effect of naringin. Flavonoids are potential opioid receptor ligands, and systemic administration of some flavonoids have been shown to elicit a dose-dependent inhibition of the nociceptive behavioral response (Alghamdi, 2020). The data indicate that naringin induced-central antinociceptive effects can be associated with the opioid receptor activation.

The potential role of serotonergic receptors in Naringin's analgesic activity has been investigated, but no relation was found with the antinociceptive effect of Naringin in hot-plate and

tail-immersion studies. It has been documented that activation of 5-HT receptors in the spinal cord causes antinociception (Sousa et al., 2017). This finding implies that naringin's antinociceptive action is not mediated by the serotonergic system.

The administration of α2-adrenoceptor agonists produce antinociception by inhibiting synaptic transmission in the dorsal horn of the spinal cord and there is evidence that stimulation of the descending noradrenergic system results in the activation of spinal α2-adrenoceptor and antinociception (Abubakar Nazifi, Odoma, Shehu, & Danjuma,, 2020). In this study, pretreatment of mice with yohimbine did not attenuate the antinociceptive activity of naringin. This implies that α2-adrenergic receptors do not play a role in its antinociceptive activity.

Modulation of nociception from acetylcholine involves the participation of multiple classes of receptors, including nicotinic and muscarinic receptors. The antinociceptive effects of acetylcholine in the dorsal horn of the spinal cord occur through mechanisms involving muscarinic receptors (M2, M3 and possibly M4) (Oliveira et al., 2018). The present results indicate that intraperitoneal administration of atropine did not impair the prolongation of reaction time induced by naringin.

Another point of this present investigation was to determine the connection of the cholinergic (nicotinic) modulation with naringin-induced antinociception. Analgesia can be achieved by influencing pathways other than the opioidergic pathways and one promising alternate avenue outside of opioid agents is to exploit the antinociceptive effects of the neuronal nicotinic acetylcholine receptors of this neurotransmitter system (Nissen et al., 2018). Mecamylamine is almost completely absorbed and readily crosses the blood–brain barrier where it acts as an acetylcholinergic nicotinic receptors antagonist (Shytle et al., 2002). In previous studies, mecamylamine administered prior to neuronal nicotinic receptor agonists prevented the antinociceptive effect in mice (Arihan, Boz, Iskit, & Ilhan,2009). In this study the antinociceptive effect of Naringin was also blocked by nicotinic receptor antagonist mecamylamine. The results indicate that there is an involvement of nicotinic receptors since mecamylamine inhibited the antinociceptive effect of naringin. Nicotinic acetylcholine receptors (major subtypes; α4β2 and α3β4) are expressed in the central nervous system, including many areas contributing to pain such as the midbrain, medulla, nucleus raphe magnus, thalamus, pedunculopontine tegmental nucleus, and spinal cord. α4β2 nicotinic full agonists were reported to display a wide-range profile of antinociceptive activity (AlSharari, Carroll, McIntosh, & Damaj, 2012).

Besides, the activation of supraspinal nicotinic acetylcholine receptors can be proved as an analgesic target for animal models of acute and chronic pain. The nicotinic acetylcholine receptors agonist ABT-594 displayed antinociceptive efficacy similar to μ-opioid receptor (MOR) agonists (Dziechciaż et al., 2013). Opioid receptors can significantly contribute to antinociception in the majority of organisms; it has been suggested that cholinergic receptor agonists effects in the analgesiometric tests are mediated via a pathway including opioid receptors. Consequently, it was believed that the co-administration with naloxone reversed the antinociceptive effects of the nicotinic receptor agonist, epibatidine, in the naked mole-rat models (Tdulu, Kanui, Towett, Maloiy, & Abelson, 2014). Similarly, the pain signaling of the dorsal horn of the spinal cord, which also expresses neuronal nicotinic receptors, emerged as a research field of interest. To conclude, the spinal neuronal nicotinic receptors have been also connected with nociceptive and antinociceptive roles (Nissen et al., 2018).

Previous studies demonstrated that daily intraperitoneal injection of naringin can promote neuroprotective roles in a rat model of Parkinson's disorder (Jung & Kim, 2014). Accordingly, the ip injection of naringin notably enhanced the glia-derived neurotrophic factor (GDNF) levels through the activation of (mTORC1) in nigral dopaminergic neurons (Leem et al., 2014; Jung, Leem, & Kim, 2014). It can be revealed that naringin increased dopamine levels in all the regions (Kola, Akula, NissankaraRao, & Danduga, 2017). On the other hand, it can be suggested that peripheral dopamine receptors might be involved in an antinociceptive action (Okumura et al., 2015).

KIR3 channels contribute to regulating postsynaptic potentials in the central and peripheral nervous systems. Besides, candidates which can regulate neuronal excitability via KIR3 channels can potently relieve the pain. It was revealed that Naringin activates KIR3 channels (Yow et al., 2011). The activation of KIR3 channels as a pervasive analgesic mechanism, in addition to opioids, mediates pain modulation. The evolution of direct KIR3 channel activators could be recommended as a potent plan to develop innovative analgesics. Thalamus and limbic cortex which express KIR3.1, 3.2, and 3.3 subunits as well as opioid receptors can be suggested as possible sites of supraspinal KIR3-mediated analgesia (Nagi et al., 2014). Thus, it can be suggested that the antinociceptive activity of naringin may be related to KIR-3 channel activity.

To summarize, various systems play a partial role in pain relief which is rational given that the central control of pain is coordinated through many neurotransmitters i.e. acetylcholine and endo-opioids. Therefore, adjunct studies evaluating the combination of several antagonists can be carried out to determine their possible involvement in pain control.

CONCLUSION

In summary, naringin presents a central antinociceptive property in mice which is possibly arranged by spinal/supraspinal-mediated opioidergic and nicotinergic modulation. It can be also assumed that the stimulation of KIR3 channels and the dopaminergic system might manage the antinociception of naringin. Consequently, naringin might act as a favorable candidate for pain relief. Additionally, the clarification of the effect and mechanisms of actions of the citrus flavonoid, naringin will contribute to new therapeutic approaches and provide guidance for new drug development studies. Therefore, this study opens up a new window for pain management using the naringin molecule. Future studies may involve clinical trials in order to clarify whether humans can be subjected to pain reduction via naringin i.p. administration.

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Author contributions: Conception/Design of Study- M.E.O.; Data Acquisition- M.E.O., Ç.K.K.; Data Analysis/Interpretation- M.E.O., Ç.K.K.; Drafting Manuscript- M.E.O.; Critical Revision of Manuscript- M.E.O., Ç.K.K.; Final Approval and Accountability- M.E.O., Ç.K.K.

Conflict of Interest: The authors have no conflict of interest to declare.

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