European Review for Medical and Pharmacological Sciences 2023; 27: 10773-10784

Exploring the therapeutic potential of fisetin: a comprehensive study on its anti-nociceptive and anti-inflammatory effects

E. QNAIS[1], A. ALQUDAH[2], Y. BSEISO[1], O. GAMMOH[3], M. WEDYAN[1], B. ALOTAIBI[4]

[1]Department of Biology and Biotechnology, Faculty of Science, The Hashemite University, Zarqa, Jordan
[2]Department of Clinical Pharmacy and Pharmacy Practice, Faculty of Pharmaceutical Sciences, The Hashemite University, Zarqa, Jordan
[3]Department of Clinical Pharmacy and Pharmacy Practice, Faculty of Pharmacy, Yarmouk University, Irbid, Jordan
[4]Department of Pharmaceutical Sciences, College of Pharmacy, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia

E. Qnais and A. Alqudah contributed equally to this work

Abstract. – OBJECTIVE: This study's primary objective was to explore and validate the pain-relieving and inflammation-reducing properties of fisetin, a flavonoid known for its antioxidant benefits, using different mouse models.

MATERIALS AND METHODS: We assessed fisetin's pain-relieving effects using mouse models exposed to both heat-induced and chemical-induced pain. The inflammation-reducing capacity of fisetin was evaluated using the carrageenan-triggered paw swelling test, focusing on the influx of leukocytes in the peritoneal space. The air pouch test was utilized to determine fisetin's ability to counteract proinflammatory cytokines. The performance of fisetin, when paired with opioid blockers, was analyzed, and juxtaposed with results from conventional medicines. The muscle-relaxing potential of fisetin was assessed through the open field assessment.

RESULTS: Fisetin consistently demonstrated marked anti-inflammatory actions across various models. It also proved to be effective in reducing pain in the pain-induced models. When combined with opioid blockers, fisetin's effects were on par with those of traditional medications. Noteworthily, fisetin displayed muscle-relaxing properties in the open-field assessment.

CONCLUSIONS: The compiled data showcases fisetin as a powerful anti-inflammatory agent with significant pain-relieving capacities, positioning it as a promising contender for pain treatment modalities.

Key Words:
Inflammation, Nociception, Fisetin, Flavonoid, Naloxone.

Introduction

Inflammation is a defense mechanism in organisms against detrimental triggers such as infections and tissue injuries[1]. However, extended immune reactions to these factors can escalate the likelihood of diseases like rheumatoid arthritis, type 2 diabetes, cancer, cirrhosis, Alzheimer's, and several neurological conditions. Pain is a typical byproduct of inflammation, emphasizing the global urgency of effective pain management. Roughly 20% of adults experience either short-term or persistent pain, affecting their overall health, daily function, financial standing, and even national economies[2,3].

Hyperalgesia emerges due to inflammatory agents that amplify the sensitivity of pain receptors and sensory neurons, leading to enhanced, prolonged pain sensations[4]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are a common global remedy for pain and inflammation relief[5]. However, extended consumption of these painkillers can induce notable adverse effects[6]. This has intensified the quest for a cost-efficient anti-inflammatory medicine with fewer side effects. As a result, the research spotlight has shifted to plant-based drugs, given their diverse molecular configurations and broad pharmacological effects[7].

Fisetin, scientifically termed 3,7,3′,4′-tetrahydroxyflavone or 7,3′,4′-flavon-3-ol, is a multifunctional flavonoid naturally present in several fruits and vegetables like grapes, apples, onions,

Corresponding Author: Abdelrahim Alqudah, Ph.D; e-mail: abdelrahim@hu.edu.jo

strawberries, and cucumbers, with varying concentrations in different plants[8]. The average daily human intake is about 0.4 mg[9]. Fisetin boasts diverse medicinal attributes, notably its inflammation-reducing effects evident from its ability to curb the release of inflammation-causing proteins triggered by lipopolysaccharides[10]. It also acts as a neuroguardian and antioxidant, primarily by stimulating the NF-E2-related factor 2/Antioxidant responsive element (Nrf2/ARE) pathway[11,12]. Additionally, fisetin has displayed anti-cancer properties and has been used in treating vascular dementia[13,14].

Despite having numerous health benefits, there has been little research to focus on fisetin's potential pain-relieving effects and anti-inflammatory actions. Hence, this study's core intent was to probe fisetin's impact on pain perception using several mouse models. We also scrutinized fisetin's inflammation-reducing attributes, examining inflammation sites for leukocyte presence and inflammation-inducing protein levels. An open-field test was undertaken to determine any behavioral shifts caused by fisetin.

Materials and Methods

Drugs

All the drugs, which include fisetin, indomethacin, naloxone, diclofenac sodium, capsaicin, formalin, morphine, carrageenan, and dexamethasone, were procured from Sigma Aldrich, Burlington, Massachusetts, USA.

Animals

The study used male Swiss Albino mice with a weight range of 20-30 g. These animals were housed in sanitized plastic enclosures and were kept under specific conditions with controlled temperature, humidity, and light-dark cycles. They had unrestricted access to food and water. Before experimentation, the mice were given 14 days to adapt to laboratory conditions. Behavioral evaluations took place between 8:00 a.m. and 12:00 p.m. after ensuring the mice were fasted overnight.

Fisetin Antinociceptive Activity

Hot plate test

The pain-relief potential of fisetin was assessed using Eddy's hot plate technique[15]. For this analysis, mice that quickly reacted, showing behaviors like jumping or withdrawal within 15 seconds when exposed to heat, were chosen. The selection process occurred a day before the actual experiment.

The test mice were categorized into ten distinct groups, with each group containing 6 mice. The first group, Group I, was given a 1% tween 80 treatment. Group VI received naloxone, a substance that counteracts opioids, combined with saline at a 2 mg dosage. Groups II, III, and IV were given fisetin in increasing doses: 25 mg, 50 mg, and 75 mg, respectively. Groups VII, VIII, and IX were treated with a combination of naloxone and varying dosages of fisetin. As a benchmark for effectiveness, Group V was given morphine, while Group X was treated with a mix of morphine and naloxone.

To ensure the safety of the mice's paws, they were placed on a 50ºC hot plate for 20 seconds. Observations of their behaviors were taken both before the drug treatments and at intervals of 30-, 60-, 90-, and 120-minutes' post-administration.

The efficiency of the treatment for each mouse was quantified using this formula: %MPE = [(Time after drug) - (Baseline time) / (Maximum allowed time) - (Baseline time)] × 100. Here, "Time after drug" is the reaction time post-medication, "Baseline time" is the initial reaction time before treatment, and "Maximum allowed time" is the utmost permitted time for the test, after which it stopped to protect the mice from potential harm.

Tail Immersion Test

The mice group from the hot plate experiment was similarly employed for the tail immersion analysis. In this procedure, when morphine was given to the mice, it delayed their reaction time in pulling their tails out from water heated to 55ºC. Conversely, naloxone negated the impact of drugs such as morphine. The study's primary goal was to gauge fisetin's pain-relieving effects, drawing a comparison to morphine, and inspecting its interaction with the opioid-blocking agent, naloxone.

Before the experiment kicked off, an evaluation was made on the mice based on how quickly they pulled their tails away; only those with reaction times between 1.5 and 2.5 seconds were selected as test subjects[16]. These mice were then pre-administered with different mixtures of fisetin, morphine, naloxone combined with fisetin, and naloxone mixed with morphine. They were then put through the tail immersion procedure. To ensure their safety, a maximum limit of 20

seconds was established for immersion. Observations on how long the mice left their tails in the heated water were made at 30-minute intervals for 2 hours.

Acetic Acid-Induced Nociception Test

For the study, the mice were categorized into five distinct groups. The inaugural group acted as a benchmark, receiving a 1% tween 80 solution. The remaining groups were administered varying compounds: three separate concentrations of fisetin (25 mg/kg, 50 mg/kg, and 75 mg/kg) and a dose of diclofenac sodium at 10 mg/kg, which stood as the positive reference[17]. Both fisetin and diclofenac sodium were given to the mice a quarter-hour before the primary test. Post this initial step, the rodents were introduced to a 1% acetic acid solution, dosed at 10 ml/kg. For the study's execution, these mice were observed in a chamber for an hour. Throughout this period, the instances of abdominal contractions in each mouse were diligently observed and noted.

Glutamate-Induced Nociception Test

The identical set of mice from the acetic acid pain induction test was later used for the glutamate pain triggering test. Before beginning, the rodents were given varying concentrations of fisetin (25 mg/kg, 50 mg/kg, and 75 mg/kg) and a dose of diclofenac sodium (10 mg/kg) about a quarter-hour before the test's start. After this preliminary step, each mouse was injected with 10 **µm** of glutamate on the underside of their left rear paw[18]. Post-injection, the mice were monitored for 15 minutes. Throughout this window, the frequency of licks on the affected paw by every mouse was meticulously observed and logged, signifying their pain response to the glutamate exposure.

Capsaicin-Induced Paw-Licking Test

To assess the pain-relieving properties of fisetin *via* the vanilloid receptor termed Transient Receptor Potential Vanilloid type-1 (TRPV1), mice were subjected to a capsaicin-triggered pain test. Before the onset of the experiment, the rodents received pre-treatments of different doses of fisetin and diclofenac sodium, half an hour before the test. For the actual procedure, each mouse's left paw was injected with 20 µl of capsaicin, dissolved in a solution comprising 5% ethanol and 95% phosphate-buffered saline (PBS), ensuring each paw got a dose of 1.6 **µg** of capsaicin[19]. Following this, the mice were observed in a designated cage for 5 minutes. The duration each mouse spent licking the affected paw was monitored, as this action reflects the nociception or pain sensitivity elicited by the capsaicin injection.

Formalin Induced Paw Licking Test

The pain-relieving potential of fisetin was examined using the Formalin-induced paw-licking test[20]. Mice were grouped into five categories and given a subcutaneous injection as pretreatment half an hour before the test. The divisions included a control group (which received 1% Tween 80), three fisetin-dosed groups (25 mg/kg, 50 mg/kg, and 75 mg/kg), and a benchmark group where mice were given 5 mg/kg of morphine. Post-pretreatment, 3% formalin was injected into the right hind paw's plantar surface of each mouse. They were then observed in a designated chamber for half an hour. The count of lickings during the initial phase (0-5 minutes), signaling neurogenic pain, and the latter phase (15-30 minutes), indicative of inflammatory pain, was meticulously noted. This process enabled the team to determine the pain-relieving attributes of fisetin and draw comparisons with morphine and the control batch.

Anti-inflammatory Effect of Fisetin

Carrageenan-induced paw edema test

The carrageenan-induced paw edema test in mice was employed to study the anti-inflammatory properties of fisetin[21]. Mice were categorized into five groups and administered various pretreatments: a control set got 1% Tween 80, three other sets were given fisetin at doses of 25 mg/kg, 50 mg/kg, and 75 mg/kg, respectively, and the fifth group (Group V) was pretreated with indomethacin (10 mg/kg) an hour before the experiment began. To trigger inflammation, a 50 ml dosage of 1% carrageenan was given to the right paw, whereas the left paw was administered a 50 ml shot of 0.9% saline solution. The swelling in both paws was gauged hourly for four hours using a digital plethysmometer.

Peritoneal Cavity Leukocyte Infiltration Test

The technique proposed by Vinegar et al[22] was utilized to observe leukocyte movement into the peritoneal cavity after carrageenan injection and to gauge the anti-inflammatory properties of fisetin. Mice were segmented into five batches and were given varying pretreatments: a benchmark group was treated with 1% Tween 80, three sub-

sets were provided fisetin at dosages of 25 mg/kg, 50 mg/kg, and 75 mg/kg, and the fifth batch (Group V) was pre-administered with morphine (5 mg/kg) a half-hour before experimentation. Carrageenan (1%), amounting to 500 μg, was intraperitoneally introduced to spark inflammation. After a lapse of 6 hours, the infiltration of leukocytes was assessed. The mice were subsequently humanely euthanized, with the peritoneal zone being rinsed using 2 ml of PBS infused with 1 mM ethylenediamine tetraacetic acid (EDTA) to extract the cells. This fluid underwent centrifugation, after which a comprehensive assessment of leukocyte types and differential cell counts was conducted, noting counts of overarching leukocytes, mononuclear entities, and polymorphonuclear entities.

Effect of Fisetin on Proinflammatory Cytokines

Mice were gently anesthetized with ether, and the fur on their backs was shaved off. To form a skin pouch, they were given subcutaneous injections of 5 ml of sterile air at the same location on two separate occasions, with a gap of three days between the injections[23]. These mice, now with pouches, were categorized into six batches for various treatments: one control group given 1% Tween 80, a separate group administered with 0.5 ml of carrageenan (labeled as Carrageenan control), three groups that were treated with carrageenan and different doses of fisetin (25 mg/kg, 50 mg/kg, and 75 mg/kg), and a positive control group which received carrageenan in combination with dexamethasone. An hour later, the mice were humanely put down through cervical dislocation. The pouch tissue was then carefully dissected open. The cavity was rinsed with 2 ml of saline, which was subsequently extracted. This extracted fluid was then centrifuged, and the cellular sediment acquired was inspected for the presence of proinflammatory markers, specifically tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6.

Open Field test

The potential sedative properties of fisetin were assessed using the open-field method. Mice were grouped and subjected to various treatments: one group was given 1% Tween 80, three others were given fisetin at varying doses (25 mg/kg, 50 mg/kg, and 75 mg/kg), and a positive control group received morphine (5 mg/kg). After allowing an hour for the treatments to take effect, the mice were individually placed in an open field box, partitioned into 25 squares, with dimensions of 50 cm × 50 cm × 50 cm. Each mouse had a 2-minute window to move around the box freely. The total number of squares a mouse entered, using all its paws, was noted to gauge its activity. Before introducing a new mouse, the open field was meticulously cleaned with a light ethanol solution to ensure a consistent environment and to avoid any biases.

Statistical Analysis

The experimental data was analyzed using GraphPad Prism software (La Jolla, CA, USA). Results were presented as the average (mean) ± the standard error of the mean (SEM). Differences between groups were determined using one-way analysis of variance (ANOVA) and further validated using Dunnet's post hoc test. Significance levels were set at $p<0.05$ and $p<0.01$.

Results

Fisetin Antinociceptive Activity

Hot plate test

This test was designed to gauge fisetin's capacity to combat pain caused by thermal stimuli. Morphine, a recognized pain alleviator, was used as a benchmark. When the opioid inhibitor naloxone was combined with fisetin, its effectiveness was further confirmed. Compared to untreated mice, fisetin notably extended the mice's reaction time, especially at a dose of 75 mg. Meanwhile, morphine-treated mice displayed significantly prolonged response durations (Table I).

Tail immersion test

As shown in Table II, mice's reaction times to heat when treated with fisetin or morphine were examined. Fisetin treatment led to a progressively prolonged reaction time, with morphine causing the most prolonged reaction. However, when naloxone was added, mice that received both fisetin and naloxone still displayed longer latency times.

Acetic acid-induced nociception test

The abdominal writhing test was used to test fisetin's pain-relieving capability. Acetic acid was administered to induce pain responses in mice, and the number of pain-induced movements was

Table I. Analysis of fisetin's pain-relieving properties and the counteractive impact of naloxone in the tail immersion pain test in mice.

Treatment (mg/kg)	Pre-treatment	Response time(s) (% MPE)			
		30 min	60 min	90 min	120 min
Control	7.05 ± 0.23	7.20 ± 0.61	7.41 ± 0.44	7.71 ± 0.32	8.17 ± 0.37
Fisetin (25 mg)	7.51 ± 0.16	8.88 ± 0.21 (11.00)	11.03 ± 0.18 (28.20)#	11.53 ± 0.63 (32.20)*	12.21 ± 0.51 (37.60)*
Fisetin (50 mg)	7.24 ± 0.23	9.32 ± 0.32 (16.30)	11.17 ± 0.41 (30.80)#	12.41 ± 0.20 (40.50)*	12.88 ± 0.25 (44.20)*
Fisetin (75 mg)	7.66 ± 0.25	10.48 ± 0.21 (22.90)	12.88 ± 0.47 (42.30)#	13.95 ± 0.85 (51.00)*	14.58 ± 0.95 (56.10)*
Morphine (5 mg)	7.20 ± 0.61	12.28 ± 0.25 (39.70)	14.66 ± 0.34 (58.30)#	15.88 ± 0.35 (67.80)*	17.65 ± 0.85 (81.60)*
NLX (2 mg) + Control	7.45 ± 0.15	7.52 ± 0.35	8.02 ± 0.52	8.32 ± 0.44	8.66 ± 0.61
NLX (2 mg) + Fisetin (25 mg)	7.41 ± 0.15	8.14 ± 0.36 (5.8)	8.55 ± 0.45 (9.10)#	9.22 ± 0.25 (14.40)*	10.19 ± 0.51 (22.10)*
NLX (2 mg) + Fisetin (50 mg)	7.19 ± 0.21	7.65 ± 0.52 (3.60)	8.58 ± 0.41 (10.90)#	9.32 ± 0.47 (16.60)*	10.44 ± 0.63 (25.40)*
NLX (2 mg) +Fisetin	7.15 ± 0.48	7.85 ± 0.23 (5.50)	8.66 ± 0.45 (11.80)#	10.41 ± 0.74 (25.40)*	11.45 ± 0.25 (33.50)*
NLX (2 mg) + Morphine (5 mg)	7.44 ± 0.17	7.75 ± 0.27 (2.50)	9.33 ± 0.25 (15.00)#	10.41 ± 0.19 (23.60)*	13.87 ± 0.55 (51.20)*

The markers "#" and "*" denote statistical differences between the control and other groups at $p < 0.05$ significance level, respectively, based on Dunnett's test. MPE: maximum possible effect; NLX: Naloxone.

counted. Mice given fisetin, especially at a 75 mg dosage, showed significantly fewer pain reactions, almost matching the effect of the benchmark drug, diclofenac sodium (Figure 1).

Glutamate-induced nociception test

Figure 2 illustrates fisetin's pain-relief performance against glutamate. Fewer licks signify better pain mitigation. Mice given 75 mg of fisetin licked their paws about as often as those treated with the benchmark, diclofenac sodium. Lower doses of fisetin also reduced licking rates compared to control mice after glutamate exposure.

Capsaicin-induced paw-licking test

Capsaicin injection led to immediate paw licking in mice, peaking within the initial 5 minutes. When compared to the benchmark, diclofenac sodium, a 75 mg dose of fisetin, caused a reduction in this behavior. The greatest licking frequency was in the control group, while lower fisetin doses still led to significantly decreased licking (Figure 3).

Formalin-induced paw-licking test

Figure 4 compares fisetin's effects to morphine in a formalin-induced pain scenario. The test,

Table II. Evaluating the pain-relieving properties of fisetin and the counteracting effects of naloxone in the tail immersion pain model in mice.

Treatment (mg/kg)		Response time(s)			
	Pre-treatment	30 min	60 min	90 min	120 min
Control	2.85 ± 0.30	3.17 ± 0.3	3.35 ± 0.20	3.53 ± 0.20	2.61 ± 0.10
Fisetin (25 mg)	2.81 ± 0.20	3.41 ± 0.30	3.57 ± 0.3	3.91 ± 0.3	4.17 ± 0.3
Fisetin (50 mg)	2.16 ± 0.3	3.71 ± 0.2#	4.21 ± 0.3*	4.80 ± 0.2*	4.91 ± 0.3*
Fisetin (75 mg)	2.17 ± 0.2	3.12 ± 0.20#	4.65 ± 0.3*	5.21 ± 0.30*	5.21 ± 0.2*
Morphine (5 mg)	2.71 ± 0.2	4.11 ± 0.4#	4.99 ± 0.3*	5.55 ± 0.2*	5.67 ± 0.2*
NLX (2 mg) + Control	2.52 ± 0.1	2.91 ± 0.2	3.11 ± 0.3	3.32 ± 0.1	3.41 ± 0.2
NLX (2 mg)+ Fisetin) (25 mg	2.65 ± 0.2	3.33 ± 0.3#	3.49 ± 0.2*	3.69 ± 0.2*	4.10 ± 0.3*
NLX (2 mg) + Fisetin (50 mg)	2.85 ± 0.3	3.55 ± 0.2#	3.67 ± 0.2*	3.99 ± 0.2b*	4.51 ± 0.3**
NLX (2 mg) + Fisetin (75 mg)	2.81 ± 0.2	3.67 ± 0.2#	3.82 ± 0.3*	4.30 ± 0.20 *	4.52 ± 0.3*
NLX (2 mg) + Morphine (5 mg)	2.01 ± 0.1	2.25 ± 0.2#	2.75 ± 0.3*	3.10 ± 0.2*	3.50 ± 0.3*

The markers "#" and "*" denote statistical differences between the control and other groups at $p < 0.05$ significance level, respectively, based on Dunnett's test. NLX: Naloxone.

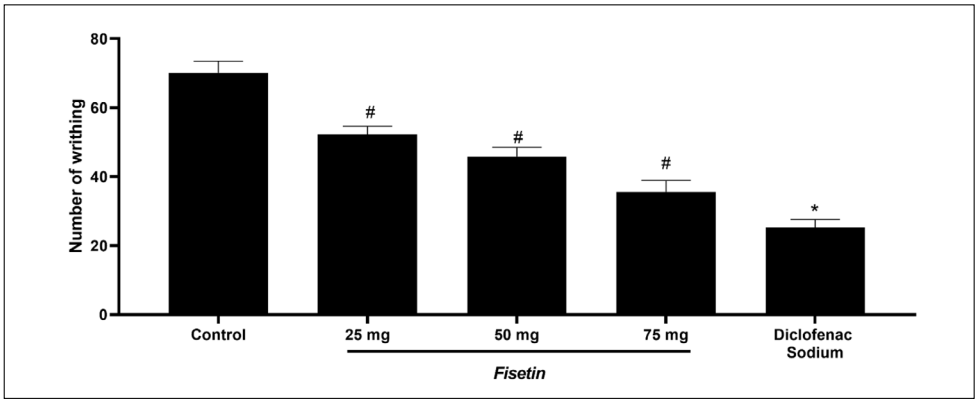

Figure 1. Pain-relieving properties of fisetin and diclofenac sodium as depicted in the acetic acid pain induction test in mice. The results are displayed in bar chart format, with each bar signifying the average ± SEM for a sample of six mice. The markers "#" and "*" denote statistical differences between the control and other groups at $p<0.05$ and $p<0.01$ significance levels, respectively, based on Dunnett's test.

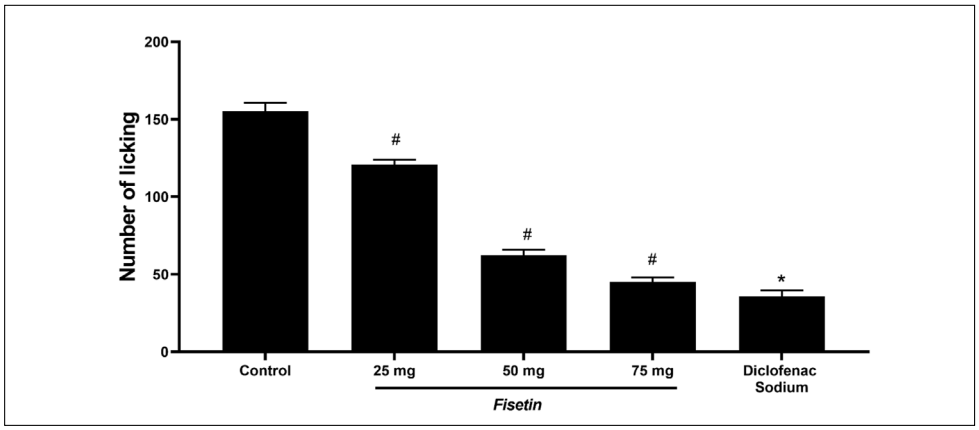

Figure 2. Evaluation of the pain-relieving impact of fisetin and diclofenac sodium in the glutamate-induced pain model in mice. Results are illustrated in bar chart format, with each bar representing the average ± SEM from a set of six mice. The markers "#" and "*" denote statistical differences between the control and the respective groups at $p<0.05$ and $p<0.01$ thresholds. These distinctions were ascertained through Dunnett's test.

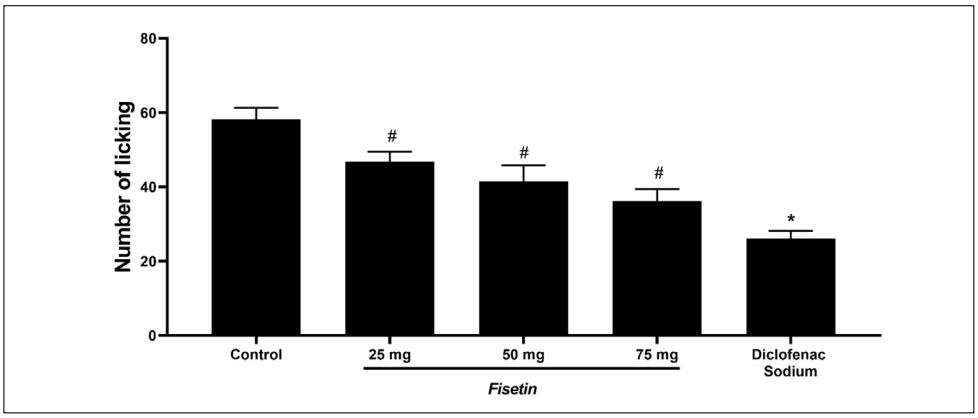

Figure 3. Comparative analysis of the pain-alleviating properties of fisetin and diclofenac sodium in the capsaicin-triggered pain model in mice. Each bar illustrates the average ± SEM from six mice. The markers "#" and "*" signify differences deemed statistically significant between the control and other groups at thresholds of $p<0.05$ and $p<0.01$, respectively, as discerned through Dunnett's test.

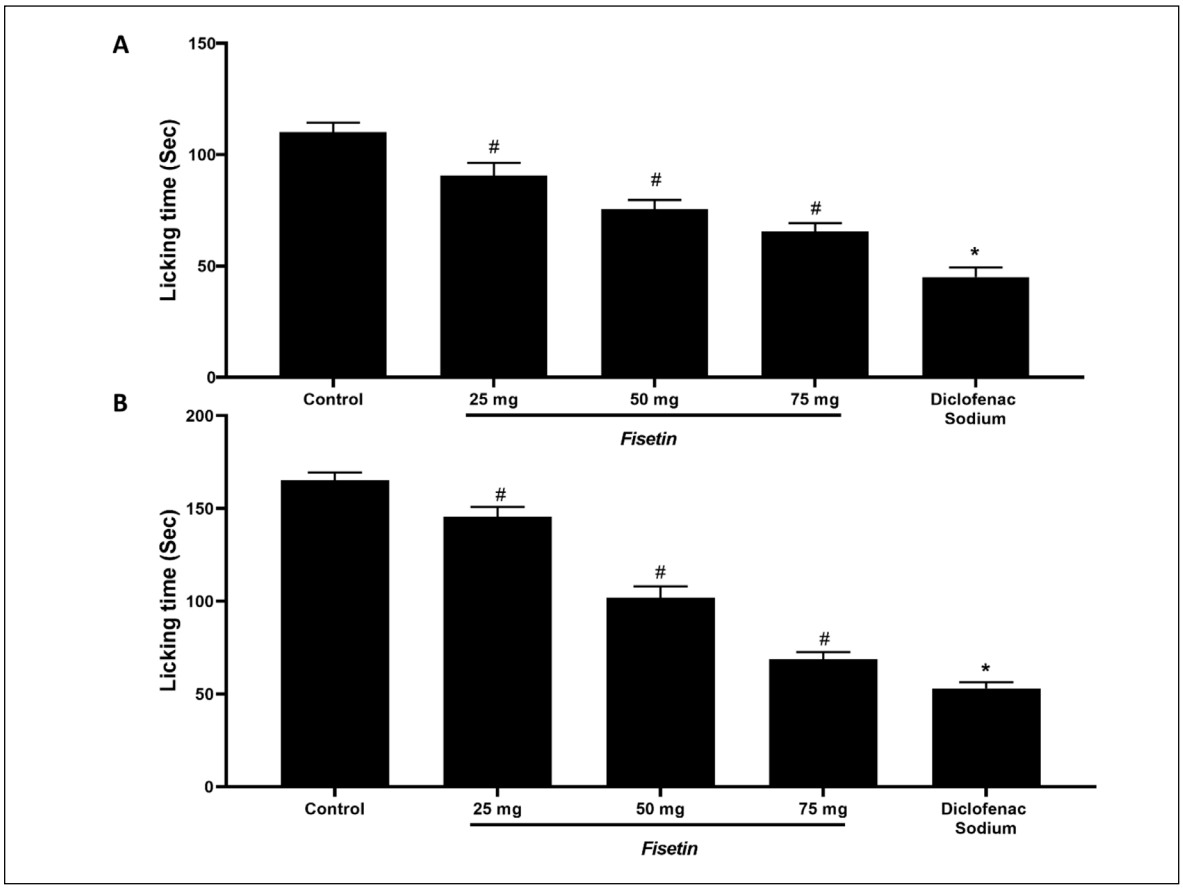

Figure 4. Evaluating the pain-relieving properties of fisetin and morphine in the biphasic formalin-triggered pain model in mice. Each bar in the graph illustrates the average ± SEM derived from six mice. The markers "#" and "*" highlight differences that are statistically significant between the control group and the other groups at thresholds of $p<0.05$ and $p<0.01$, respectively, based on Dunnett's test results.

conducted in two stages, was designed to validate fisetin's pain-relief efficacy. In both test phases, fisetin reduced paw-licking behavior compared to untreated mice. It is worth highlighting that in the later phase (15-30 minutes), fisetin-treated mice licked their paws more frequently than in the earlier phase (5 minutes). In contrast, morphine outperformed both the control and fisetin-treated groups.

Anti-Inflammatory Effect of Fisetin

Carrageenan-induced paw edema test

This test measured the inflammation-reducing capabilities of fisetin against carrageenan-induced paw swelling. Hourly measurements of paw size revealed that both fisetin (especially at the 75 mg dose) and indomethacin (a known anti-inflammatory drug) caused a decrease in paw swelling by the 4th hour. This suggests that fisetin can inhibit carrageenan-induced inflammatory responses comparably to indomethacin, underlining its potential anti-inflammatory benefits (Table III).

Peritoneal Cavity Leukocyte Infiltration Test

Figure 5 presents the assessment of leukocytes, both mononuclear and polymorphonuclear, that have infiltrated the peritoneal cavity of mice. Mice receiving fisetin (all doses) or morphine showed decreased leukocyte infiltration compared to the carrageenan-only group. Interestingly, the morphine group had the lowest levels of leukocyte infiltration, highlighting its strong anti-inflammatory effect. However, the 75 mg fisetin dosage also showcased significantly reduced leukocyte infiltration, rivaling the effect of morphine. This highlights that fisetin, especially at a 75 mg dose, may be as efficient as morphine in reducing leukocyte infiltration.

Table III. Comparison of the anti-inflammatory properties of fisetin and indomethacin in the carrageenan-triggered inflammation model in mice.

Treatment (mg/kg)	Basal	Response time(s)			
		1st h	2nd h	3rd h	4th h
Control	24.8 ± 2.1	142.5 ± 7.9	132.7 ± 6.4	126.6 ± 5.2	118.2 ± 4.3
Fisetin (25 mg)	25.4 ± 4.4	95.4 ± 5.2	92.1 ± 3.8#	90.2 ± 3.7*	82.4 ± 4.6*
Fisetin (50 mg)	26.20 ± 1.7	91.3 ± 3.2	89.6 ± 6.4#	80.1 ± 6.5*	77.2 ± 3.3*
Fisetin (75 mg)	28.2 ± 2.7	88.4 ± 6.4	76.3 ± 3.5#	63.3 ± 4.2*	59.7 ± 4.8*
Indomethacin (10 mg)	24.6 ± 3.7	73.5 ± 2.8	72.7 ± 4.2#	65.6 ± 4.8*	60.4 ± 2.9*

The markers "#" and "*" denote statistical differences between the control and other groups at $p < 0.05$ significance level, respectively, based on Dunnett's test. h: hour.

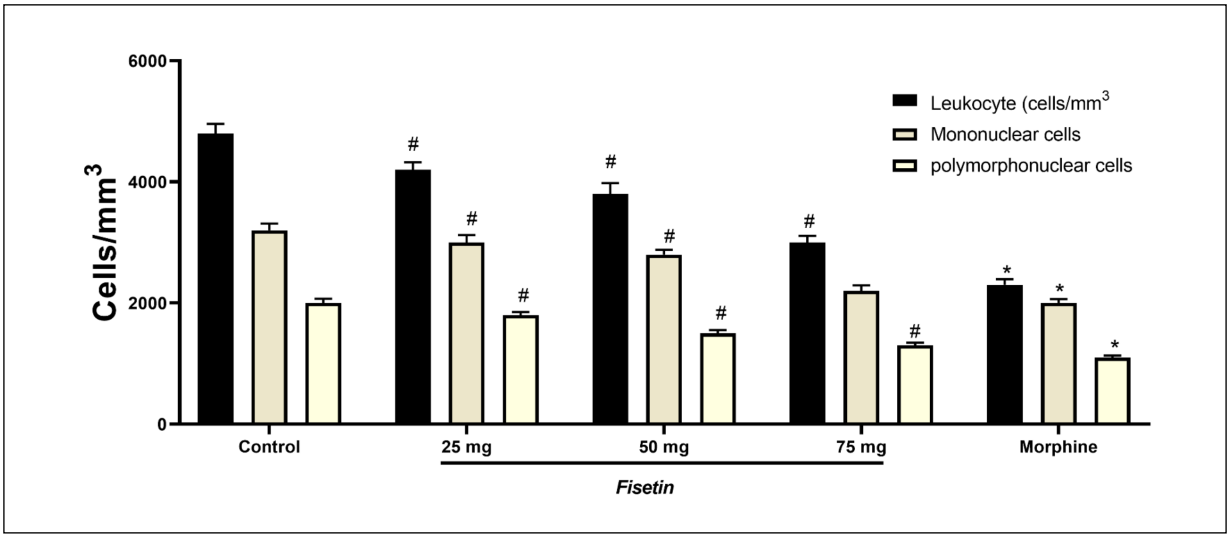

Figure 5. Comparative analysis of the anti-inflammatory actions of fisetin and morphine on leukocyte infiltration in the peritoneal cavity in mice subjected to carrageenan-induced inflammation. Each bar graph illustrates the mean ± SEM for six mice. Symbols "#" and "*" denote statistically significant differences between the control group and other groups at significance levels of $p<0.05$ and $p<0.01$, respectively, as determined by Dunnett's test.

Effect of Fisetin on Proinflammatory Cytokines

The graphics in Figure 6A, Figure 6B, and Figure 6C showcase the levels of three proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in carrageenan-induced air pouches of mice. Mice treated with carrageenan alone had notably higher IL-6 concentrations, while fisetin-treated and dexamethasone-treated mice exhibited significant reductions in TNF-α levels (Figure 6A). This suggests that fisetin and dexamethasone can effectively inhibit the rise of proinflammatory cytokine TNF-α, showcasing their anti-inflammatory potential.

Open Field Test

The test assessed the sedative properties of fisetin by observing mouse behavior in an open field. While the 25 mg and 50 mg doses of fisetin did not lead to any significant behavioral change compared to control mice, a decrease in activity was observed in mice treated with 75 mg of fisetin and morphine, as indicated by fewer crossed squares in the open field apparatus (Figure 7).

Discussion

In this study, we examined how the dose of fisetin affected its pain-relieving and anti-inflam-

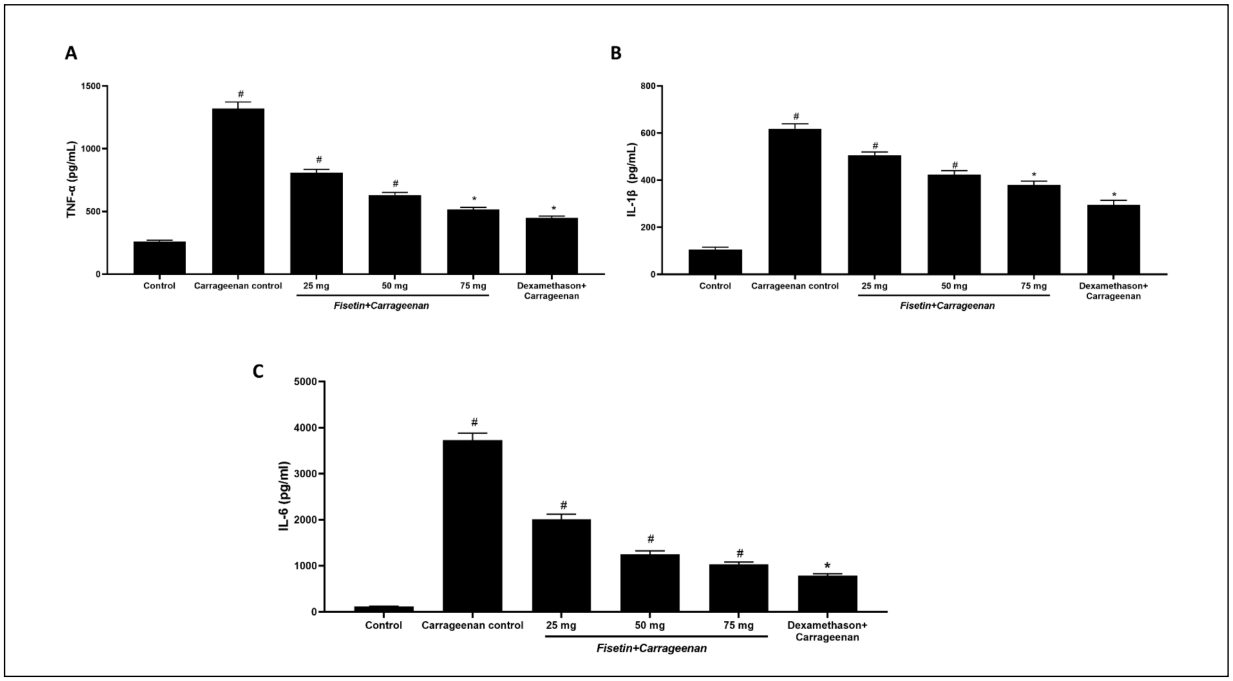

Figure 6. Evaluation of anti-inflammatory properties of fisetin and morphine in relation to proinflammatory cytokine levels using the air pouch model in mice. Each bar depicts the mean ± SEM for a group of six mice. Symbols "#" and "*" highlight the differences deemed statistically significant between the control and other groups at significance thresholds of $p<0.05$ and $p<0.01$, respectively, following Dunnett's test analysis.

matory properties using various *in vivo* models. We compared fisetin's effects to standard drugs and also observed the counteracting effects when combined with the opiate blocker, naloxone. Fisetin's anti-inflammatory abilities were gauged by looking at leukocyte movement in the peritoneal area and checking the levels of inflammation-causing agents.

Nociception, the body's reaction to harmful stimuli, was tested to determine the drug's po-

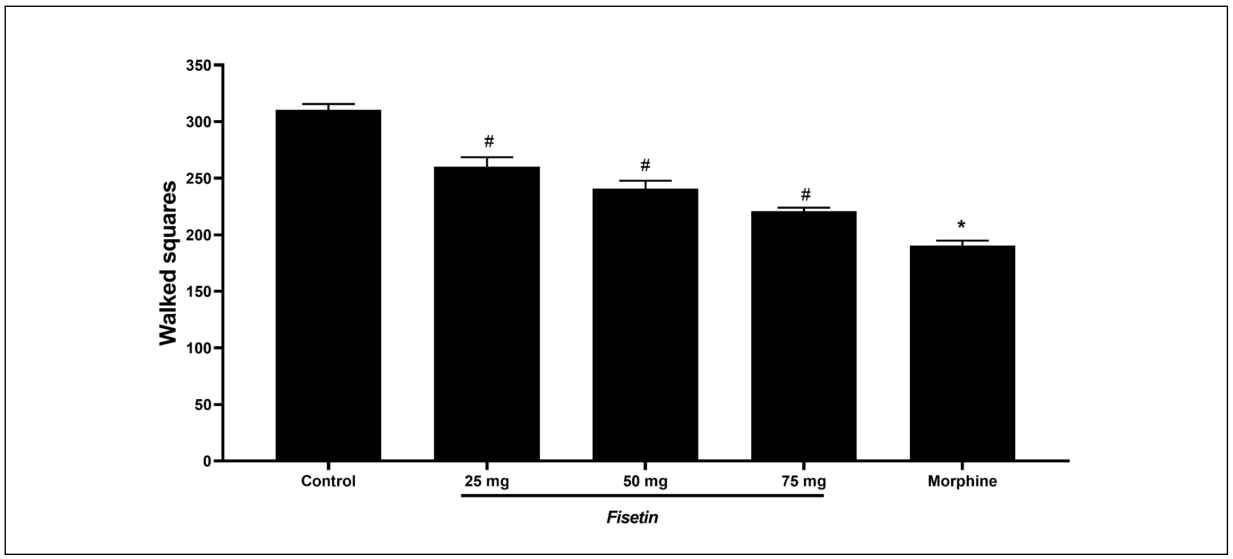

Figure 7. Behavioral assessment of mice in the open field following treatment with fisetin and morphine. Bar graphs illustrate the data, with each bar denoting the mean ± SEM for a set of six mice. Symbols "#" and "*" represent statistical differences between the control and other groups at significance levels of $p<0.05$ and $p<0.01$, respectively, based on Dunnett's test analysis.

tential to alleviate pain[24]. This involved using heat, pressure, or electric shock. When subjected to heat through a hot plate and tail immersion tests[25,26], fisetin, given at 75 mg/kg body weight, notably prolonged the mice's reaction time, similar to the standard drug, morphine. This might be because fisetin interacts with the pain reflexes in the spinal cord and brain through opioid receptors. This idea was further supported when the effects of fisetin were partially reversed using naloxone, an opioid blocker.

Pain receptor sensitivity can be gauged by introducing irritants like acetic acid, which causes mice to writhe. The test was conducted to evaluate the ability of the pain-relief drug to block signals, specifically, those amplified by prostaglandins in the central nervous system, which heighten the sensitivity of pain receptors[27]. In tests, mice given fisetin showed fewer writhing reactions, indicating that fisetin may suppress pain-signalling chemicals like prostaglandins.

When assessing fisetin against pain caused by excitatory amino acids, a test involving glutamate in mice was done. Glutamate and aspartate are principal amino acids influencing pain perception. Glutamate acts through two main receptors in the peripheral and spinal nervous system: N-methyl-D-aspartate (NMDA) and non-NMDA[28]. Additionally, it prompts peripheral neurons to release inflammation-causing agents. In this research, mice treated with fisetin showed a reduction in licking, a sign of glutamate-caused pain, suggesting fisetin might block both NMDA and non-NMDA receptors, thus reducing pain onset.

The study examined fisetin's impact on certain pain behaviors like biting and flinching using a capsaicin-induced mouse model. This method determines the ability of a drug to counteract chemically induced pain[29]. Mice pre-treated with fisetin displayed notably fewer licks when exposed to capsaicin, suggesting that fisetin blocks the agents causing inflammation, thereby alleviating pain. Furthermore, when tested with the formalin nociception mouse model, where pain is initiated by inflammatory agents acting on sensory neurons[30], fisetin considerably reduced licking in both the neurological and inflammatory stages. This supports fisetin's efficacy as a pain-relieving drug.

In the study, fisetin was observed to counteract the carrageenan-triggered rise of leukocytes in the peritoneal cavity. This spike in leukocytes might stem from the Myeloperoxidase activity in the swelling of the paw, a result of Reactive Oxygen Species (ROS) produced by carrageenan. Fisetin likely neutralizes ROS due to its antioxidant characteristics, thus restricting the surge in leukocyte infiltration. Key inflammatory mediators such as TNF-α and IL-1β, produced by macrophages, play pivotal roles in inflammation[31]. Moreover, spinal glial cells release strong inflammatory cytokines like TNF-α, IL-1β, and IL-6, which heighten the sensitivity of pain receptors[32-34]. The study further validated fisetin's anti-inflammatory properties by using the air pouch model test, revealing that mice pre-treated with fisetin showed reduced levels of these inflammatory cytokines compared to the control group treated with carrageenan.

Lastly, the open field test was conducted to evaluate how fisetin influenced mice behavior. Outcomes from this test indicated that mice treated with fisetin performed superiorly compared to those treated with morphine. This suggests fisetin might be a powerful pain-relief drug with fewer adverse reactions.

Conclusions

The cumulative results from different pain and inflammation mouse studies clearly show that fisetin has significant pain-relieving and anti-inflammatory effects. The reduced levels of proinflammatory cytokines and the behavior of fisetin-treated mice further validate its potential as an effective anti-inflammatory agent. Importantly, fisetin seems to offer these advantages without apparent adverse effects. In summary, the detailed findings from the research suggest that fisetin is a promising, potent, and safe treatment choice for addressing pain and inflammation-related issues.

Conflict of Interest

The authors declare that they have no conflict of interests.

Authors' Contribution

Conceptualization, E.Q. and A.A.; methodology, Y.B.; software, O.G.; validation, M.W., E.Q. and O.G.; formal analysis, B.O.; investigation, A.A.; resources, E.Q.; data curation, B.O.; writing-original draft preparation, Y.B.; writing-review and editing, A.A.; visualization, M.W.; supervision, E.Q.; project administration, A.A.; funding acquisition, B.O. All authors have read and agreed to the published version of the manuscript.

Acknowledgment
This research was funded by Princess Nourah bint Abdulrahman University Researchers supporting Project number (PNURSP2023R73), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Ethics Approval
All the animal procedures were approved by the Ethical Committee of The Hashemite University (IRB #: 9/5/2019/2020).

Informed Consent
Not applicable.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

ORCID ID
Abdelrahim Alqudah: 0000-0003-3721-8225
Esam Qnais: 0000-0002-6203-4527
Yousra Bseiso: 0000-0002-4346-5049
Omar Gammoh: 0000-0001-8801-2652
Mohammed Wedyan: 0000-0002-8693-5506
Badriyah Alotaibi: 0000-0002-4066-5694

References

1) Wang J, Liu YT, Xiao L, Zhu L, Wang Q, Yan T. Anti-Inflammatory Effects of Apigenin in Lipopolysaccharide-Induced Inflammatory in Acute Lung Injury by Suppressing COX-2 and NF-kB Pathway. Inflammation 2014; 37: 2085-2090.
2) Olesen AE, Andresen T, Staahl C, Drewes AM. Human Experimental Pain Models for Assessing the Therapeutic Efficacy of Analgesic Drugs. Pharmacol Rev 2012; 64: 722-779.
3) Goldberg DS, McGee SJ. Pain as a global public health priority. BMC Public Health 2011; 11: 1-5.
4) Gudes S, Barkai O, Caspi Y, Katz B, Lev S, Binshtok AM. The role of slow and persistent ttx-resistant sodium currents in acute tumor necrosis factor- α-mediated increase in nociceptors excitability. J Neurophysiol 2015; 113: 601-619.
5) Jones R, Rubin G, Berenbaum F, Scheiman J. Gastrointestinal and Cardiovascular Risks of Nonsteroidal Anti-inflammatory Drugs. Am J Med 2008; 121: 464-474.
6) Xue N, Wu X, Wu L, Li L, Wang F. Antinociceptive and anti-inflammatory effect of Naringenin in different nociceptive and inflammatory mice models. Life Sci 2019; 217: 148-154.
7) Tsuchiya H. Anesthetic Agents of Plant Origin: A Review of Phytochemicals with Anesthetic Activity. Mol 2017; 22: 1369.
8) Pal HC, Pearlman RL, Afaq F. Fisetin and its role in chronic diseases. Adv Exp Med Biol 2016; 928: 213-244.
9) Mehta P, Pawar A, Mahadik K, Bothiraja C. Emerging novel drug delivery strategies for bioactive flavonol fisetin in biomedicine. Biomed Pharmacother 2018; 106: 1282-1291.
10) Park HH, Lee S, Oh JM, Lee MS, Yoon KH, Park BH, Kim JW, Song H, Kim SH. Anti-inflammatory activity of fisetin in human mast cells (HMC-1). Pharmacol Res 2007; 55: 31-37.
11) Naeimi AF, Alizadeh M. Antioxidant properties of the flavonoid fisetin: An updated review of in vivo and in vitro studies. Trends Food Sci Technol 2017; 70: 34-44.
12) Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. Free Radic Res 2009; 39: 1119-1125.
13) Sun X, Ma X, Li Q, Yang Y, Xu X, Sun J, Yu M, Cao K, Yang L, Yang G, Zhang G, Wang X. Anti-cancer effects of fisetin on mammary carcinoma cells via regulation of the PI3K/Akt/mTOR pathway: In vitro and in vivo studies. Int J Mol Med 2018; 42: 811-820.
14) Hemanth Kumar B, Arun Reddy R, Mahesh Kumar J, Dinesh Kumar B, Diwan P V. Effects of fisetin on hyperhomocysteinemia-induced experimental endothelial dysfunction and vascular dementia. Can J Physiol Pharmacol 2016; 95: 32-42.
15) Turner R. Screening Methods in Pharmacology. ACCADEMIC PRESS New York and London - Google Books. Available at: https://books.google.it/books?id=KhIIBQAAQBAJ&printsec=frontcover&source=gbs_atb&redir_esc=y#v=onepage&q&f=false.
16) Uma DP, Ganasoundari A, Rao BSS, Srinivasan KK. In Vivo Radioprotection by Ocimum Flavonoids: Survival of Mice. Radiat Res 1999; 151: 74-78.
17) Koster R, Anderson M, De Beer E. Acetic acid for analgesic screening. Fed Proc 1959; 18: 412.
18) Beirith A, Santos ARS, Calixto JB. Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. Brain Res 2002; 924: 219-228.
19) Luiz AP, Moura JDÁ, Meotti FC, Guginski G, Guimarães CLS, Azevedo MS, Rodrigues ALS, Santos ARS. Antinociceptive action of ethanolic extract obtained from roots of Humirianthera ampla Miers. J Ethnopharmacol 2007; 114: 355-363.
20) Hunskaar S, Fasmer OB, Hole K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods 1985; 14: 69-76.
21) Morris C. Carrageenan-Induced Paw Edema. Inflamm Protoc 2003; 225: 115-121.

22) Vinegar R, Truax JF, Selph JL. Some Quantitative Temporal Characteristics of Carrageenin-Induced Pleurisy in the Rat. Proc. Soc Exp Biol Med 1973; 143: 711-714.

23) Edwards JCW, Sedgwick AD, Willoughby DA. The formation of a structure with the features of synovial lining by subcutaneous injection of air: An in vivo tissue culture system. J Pathol 1981; 134: 147-156.

24) Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev 2001; 53: 597-652.

25) Srinivasan K, Muruganandan S, Lal J, Chandra S, Tandan SK, Raviprakash V, Kumar D. Antinociceptive and antipyretic activities of Pongamia pinnata leaves. Phyther Res 2003 17: 259-264.

26) Hiruma-Lima CA, Gracioso JS, Bighetti EJB, Germonsén Robineou L, Souza Brito ARM. The juice of fresh leaves of Boerhaavia diffusa L. (Nyctaginaceae) markedly reduces pain in mice. J Ethnopharmacol 2000; 71: 267-274.

27) Gawade SP. Acetic acid induced painful endogenous infliction in writhing test on mice. J Pharmacol Pharmacother 2012; 3: 348.

28) Dobrek Ł, Thor P. Glutamate NMDA receptors in pathophysiology and pharmacotherapy of selected nervous system diseases. Postepy Hig Med Dosw 2011; 65: 338-346.

29) Sawynok J, Reid A, Meisner J. Pain Behaviors Produced by Capsaicin: Influence of Inflammatory Mediators and Nerve Injury. J Pain 2006; 7: 134-141.

30) Vahdati Hassani F, Rezaee R, Sazegara H, Hashemzaei M, Shirani K, Karimi G. Effects of silymarin on neuropathic pain and formalin-induced nociception in mice. Iran J Basic Med Sci 2015; 18: 715.

31) Sergerie Y, Rivest S, Boivin G. Tumor Necrosis Factor-α and Interleukin-1β Play a Critical Role in the Resistance against Lethal Herpes Simplex Virus Encephalitis. J Infect Dis 2007; 196: 853-860.

32) Schomberg D, Olson JK. Immune responses of microglia in the spinal cord: Contribution to pain states. Exp Neurol 2012; 234: 262-270.

33) Taves S, Berta T, Chen G, Ji RR. Microglia and spinal cord synaptic plasticity in persistent pain. Neural Plast 2013; 2013: 1-10.

34) Kali Z, Cagiran FT. Surgical removal of intramural fibroids improves the TNF-α induced inflammatory events in endometrium. Eur Rev Med Pharmacol Sci 2022; 26: 9180-9186.