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Analgesic effect of the flavonoid herbacetin in nociception animal models

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Abstract. – OBJECTIVE: This study aimed to assess the antinociceptive activity of herbacetin using chemically and thermally induced nociception in a mouse model.

MATERIALS AND METHODS: The antinociceptive effects of various herbacetin doses (50, 100, 150, and 200 µg/kg) were assessed in mice using the acetic acid-induced writhing test, hot plate test, and formalin-induced paw-licking assay. The effects were compared to those of mice treated with acetylsalicylic acid or morphine in the presence or absence of naloxone (an opioid receptor antagonist). Capsaicin- and glutamate-induced paw-licking tests were also used to evaluate the involvement of the vanilloid and glutamatergic systems, respectively. Pro-inflammatory mediators: Interleukin-1-beta (IL-1β), Tumour Necrosis Factor alpha (TNF-α), Interferon-gamma (IFN-γ), and Nitric Oxide (NO) were also assessed.

RESULTS: Herbacetin produced significant dose-dependent inhibition of nociceptive behavior in the acetic acid-induced writhing test, showing 65% inhibition at a dose of 200 µg/kg. Herbacetin also caused a significant increase in the latency period in response to the hot plate test (70% at 200 µg/kg), and significantly inhibited both the neurogenic and inflammatory phases in the formalin-induced paw-licking test. Naloxone significantly reverses the effect of herbacetin in both the hot plate and formalin-induced paw-licking test. Moreover, herbacetin significantly inhibited the neurogenic nociception induced by intraplantar injections of capsaicin and glutamate (75% and 48%, respectively, at a dose of 200 µg/kg). Pro-inflammatory cytokines IL-1β, TNF-α, IFN-γ, and NO in the serum of mice were assessed. These cytokines were significantly inhibited by herbacetin (100 and 200 µg/kg). Thus, herbacetin exhibited peripheral and central antinociception through the modulation of vanilloid receptors, opioid receptors, and the glutamatergic system.

CONCLUSIONS: Herbacetin possesses antinociceptive activity in adult mice that is mediated through both central and peripheral pathways.

Key Words:
Herbacetin, Nociception, Flavonoids, Naloxone, Vanilloid, Glutamate.

Introduction

Pain is an unpleasant sensation that may be caused by damage to body tissues or is associated with this damage[1]. Most scientists concur that pain, whether brought on by actual or potential tissue damage, aids in preventing additional injury to the organism. Nociceptive pain, which typically happens when nociceptors are activated by extreme cold, high heat, hard mechanical stimuli, or a range of chemical stimuli, is one of the two primary types of pain brought on by harmful stimuli to the bodily tissues[2]. Understanding the fundamental mechanisms of pain, including the neurotransmitter systems, neuromodulators, ion channels, and receptors involved in the pain neural pathways, has received a lot of interest in recent years[3,4].

The presence of pain has numerous negative effects, including reducing the overall quality of life, decreasing productivity, increasing absenteeism from work, causing disability, and even leading to unemployment in up to one-third of the population. As a result, the annual cost associated with these outcomes is estimated to be in the billions of dollars[5]. Despite the high cost of treatment, predicting the effectiveness of analgesics for individual patients is difficult and often falls short of expectations for pain relief. This could

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be partly explained by the side effects of many drugs, which may discourage patients from adhering to physicians' recommendations.

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to relieve pain brought on by inflammation. However, their prolonged usage has been associated with a number of serious negative consequences, such as bleeding, peptic ulcers, and gastrointestinal lesions[6]. Despite being a frequently used therapeutic option, these risks highlight the need for caution and close monitoring when prescribing NSAIDs for extended periods.

Similarly, opioids are a significant class of analgesics that are often used to treat short-term post-operative or neurogenic pain. Opioids, however, have a number of serious adverse effects, such as constipation, respiratory depression, and tolerance[7]. Alternative therapeutic agents, such as plant-based pharmaceuticals, are therefore receiving more attention as prospective therapy choices because they have a milder action and fewer adverse effects.

Various models can be used to evaluate the antinociceptive properties of a compound. The acetic acid-induced writhing test is one such model that induces activation of a non-selective ion channel in peripheral nociceptive fibers[8]. In addition to this direct activation, acetic acid also indirectly stimulates the release of multiple autacoid mediators, including serotonin, histamine, bradykinin, and substance P, and increases levels of prostaglandins (E2 and F2)[9]. Additionally, acetic acid triggers the synthesis of lipoxygenase, which in turn causes the activation of peripheral nociceptive neurons. Furthermore, another model used to assess antinociceptive effects is the hot plate test[10]. In this test, stimulation of a hot plate induces paw licking and jumping behavior at the supraspinal level, and the duration of these responses can be used to evaluate animal behavior. The latency period of mice in the hot plate test can be prolonged only by centrally-acting opioid-like medications; peripherally-acting medicines cannot. An additional model for evaluating antinociceptive effects is the formalin-induced paw-licking test[11]. Following formalin injection, this test shows two separate periods of nociceptive behavior. Within seconds of formalin injection, the first phase begins and lasts for around five minutes. This is referred to as the neurogenic phase, and it is brought on by formalin's direct interaction with pain receptors. The second phase, known as the inflammatory phase, starts 15 to 30 minutes after formalin administration and is brought on by the production of inflammatory mediators like histamine, prostaglandins, and bradykinins[12]. Another model utilized in pain research is the capsaicin-induced paw-licking test. This test brings on both analgesia and hyperalgesia. Chili pepper constituent capsaicin activates the transient receptor potential vanilloid 1 (TRPV1) receptor, causing an influx of calcium ions (Ca2+) and sodium ions (Na+), primarily Ca2+, into afferent neurons. This activation subsequently stimulates C- or Aδ-fibers, resulting in neurogenic pain[13-15]. Additionally, a glutamate-induced paw-licking test is another model used in pain research. Glutamate is a crucial excitatory amino acid and neurotransmitter that is abundant in the central nervous system and essential for many physiological and pathophysiological processes[16]. Glutamate receptors are found in both the central and peripheral nervous systems, and their activation leads to nociceptive transmission, making them relevant to the study of pain.

Due to their abundant occurrence in plants and considerable pharmacological activity, flavonoids have attracted the attention of researchers. Studies[17] have shown that flavonoids are involved in inflammation and antinociception. It has been discovered that a number of flavonoids bind to opioid and gamma-amino butyric acid-A (GABAA) receptors, producing strong antinociceptive effects in a variety of pain models. This analgesic effect may be brought on either directly by inhibiting peripheral afferent pathways or inadvertently by increasing traffic on descending pathways[18,19].

A flavonoid molecule called herbacetin (3,4,5,7,8-pentahydroxyflavone) has been isolated principally from the plants *Rhodiola rosea* L. and *Ephedrae herba* L. Anti-diabetic, anti-cancer, antiviral and possible neuroprotective actions are documented therapeutic effects of herbacetin[20]. In addition, several studies[21-24] have reported antioxidant effects for herbacetin. Oxidative stress can have detrimental effects on cell structures, proteins, lipids, and DNA, leading to various diseases, including cancer[21], diabetes[22], atherosclerosis[23], and neurodegenerative diseases[24]. Furthermore, herbacetin exhibited potent free radical scavenging activity and effectively inhibited protein oxidation[25]. Additionally, it was found to protect against oxidative damage and carbonylation induced by Cu2+-H2O2 or 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) in a concentration-dependent manner. Herbacetin displays antiviral properties and shows promise as a

potential antiviral agent. Studies[26] have reported that herbacetin effectively reduced the cytopathic effects (CPEs) induced by H1N1 (A/PR/8/34) and H9N2 (A/Chicken/Korea/MS96/96) viruses in Madin-Darby canine kidney cells. Moreover, Jo et al[27] demonstrated that herbacetin effectively inhibited the enzymatic activity of the viral protease SARS-CoV 3CLpro. The inhibition occurred through herbacetin's binding to the His41 and Gln189 residues in the S2 site of the viral protease. Furthermore, herbacetin has been reported to exhibit anti-cancer properties, including the inhibition of tumor-cell metastasis, induction of apoptosis, and display of cytotoxic effects. Hyuga et al[28] found that herbacetin could suppress the motility of MDA-MB-231 human breast cancer cells induced by hepatocyte growth factor (HGF) by inhibiting c-Met and AKT phosphorylation, without affecting cell viability. The study also demonstrated that herbacetin effectively inhibited c-Met tyrosine kinase activity *in vitro* in a concentration-dependent manner.

Moreover, the anti-diabetic effect of herbacetin was also reported. Herbacetin has demonstrated its potential in improving high-fat-diet-induced type 2 diabetes (T2D) in C57BL/6 J mice by positively impacting insulin levels and regulating liver lipid metabolism. This regulation involves the modulation of lipid regulatory enzymes, including fatty acid synthase (FAS), carnitine palmitoyltransferase (CPT), fatty acid β-oxidation (β-oxidation), glucose 6-phosphate dehydrogenase (G6PD), and sterol regulatory element-binding proteins (SREBPs) 1c and 2[29]. In the context of diabetes treatment, an important target is fructose-1,6-bisphosphatase (FBPase), which plays a regulatory role in the gluconeogenesis pathway. Proença et al[30] have reported that herbacetin effectively inhibits human FBPase by forming a stable interaction with the Glu30 side chain and the Thr24 backbone of the enzyme.

Previous studies[31] have reported that herbacetin exhibits anti-inflammatory properties by reducing the expression of pro-inflammatory cytokines, demonstrating antioxidant activity, and inhibiting nuclear factor-κB (NF-κB) and inducible nitric oxide synthase (iNOS) in various systems. It also suppresses the production of prostaglandin E2 and has the potential to alleviate neurodegenerative diseases. However, no study has investigated the role of herbacetin in pain treatment[32].

By using a variety of nociception models, such as the acetic acid-induced writhing test, hot plate test, formalin-induced paw-licking test, capsaicin-induced paw-licking test, and glutamate-induced paw-licking test, the current study aimed to investigate the antinociceptive potential of herbacetin in male mice. Additionally, the study sought to determine if any potential mechanisms of antinociception exhibited by herbacetin could be identified through conducting different nociception behavioral tests.

Materials and Methods

Experimental Animals

This study was conducted using adult male Swiss albino mice that were bred and raised at the Animal House Unit of the Hashemite University and weighed between 25-29 g. The necessary approvals for animal care and experimentation procedures were obtained from the Animal Research Ethics Committee at Hashemite University (IRB number: HU 104/2022), and all procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals[33].

The mice were maintained in a regulated environment with a temperature of 20±2°C and a 12-hour light/dark cycle where the lights were turned on in a range of 6 to 18 hours per day. They were provided with unrestricted access to food and water. To minimize stress, the mice were transferred to the testing area an hour before the experiments to adapt to the laboratory settings. During the animal experiments, two trained observers, who were not informed about the experimental design, observed the mice to avoid bias. The experiments were carried out in a room that was soundproofed.

Acetic Acid-Induced Writhing Test

Six groups of mice were given intraperitoneal treatment according to the previously described method[34]. The first group, which served as a control, received a solution consisting of 5% dimethylsulfoxide (DMSO) and 95% distilled water. The remaining four groups were given herbacetin (SMB00355, Sigma-Aldrich, Merck, Burlington, MA, USA) in doses of 50, 100, 150, and 200 µg/kg, respectively, in the same vehicle solution. The sixth group received a dosage of 100 mg/kg of acetylsalicylic acid (A5376, Sigma-Aldrich, Merck, Burlington, MA, USA). The doses of herbacetin were chosen based on prior pilot experiments. After 60 minutes, a dose of 10 mL/kg of body weight of acetic acid 0.6% (A6283, Sigma Aldrich, Mer-

ck, Burlington, MA, USA) was administered to each mouse, following the vehicle, herbacetin, or acetylsalicylic acid (ASA) treatments.

During this experiment, the researchers observed the mice for a period of 30 minutes after administering acetic acid and recorded the number of complete writhing events. Complete writhing was defined as body elongation, abdominal contraction, twisting of the pelvis, and/or trunk twisting accompanied by the extension of limbs.

To determine the percentage of inhibition of writhing (PIW), the following formula: % Inhibition = [(C - T) / C] x 100 was used. Here, C represents the number of writhing episodes observed in the control group, and T represents the number of writhing episodes observed in the treated group.

Hot Plate Test

Eight groups of mice were used in this test. The vehicle control group, group one, was given a solution consisting of 5% DMSO in distilled water. Groups two to five were treated with herbacetin at doses of 50, 150, 150, and 200 µg/kg, respectively. Group seven received Morphine (M8777, Sigma Aldrich, Merck, Burlington, MA, USA) that was dissolved in sterile saline and administered intraperitoneally at a dosage of 5 mg/kg. To test for the involvement of the opioidergic system, groups six and eight received naloxone hydrochloride (BP548, Merck, Burlington, MA, USA), a non-selective opioid receptor antagonist, at a dosage of 5 mg/kg intraperitoneally, 15 minutes prior to treatment with herbacetin (200 µg/kg) and morphine (5 mg/kg i.p.), respectively. All treatments were administered intraperitoneally and were given 60 minutes before subjecting the mice to testing with the analgesiometer hot plate at a temperature of 55±5°C. In order to determine the reaction time of the mice, we recorded the time interval between placing the animal on the hot plate and when it started to paw lick. The reaction time was measured twice: first before treatment and then again 60 minutes after treatment. The results were expressed as the percentage increase in baseline, which was calculated using the following formula: percentage increase in baseline = [(A-B) / B] × 100. Here, A represents the reaction time after treatment, and B represents the reaction time before treatment[35].

Formalin-Induced Paw Licking Test

Nine groups of mice were used. The first group, referred to as the vehicle control, was given distilled water containing 5% DMSO. The second to fifth groups were treated with herbacetin at doses of 50, 100, 150, and 200 µg/kg, respectively. The sixth group was administered morphine (5 mg/kg), while the seventh group received ASA (100 mg/kg).

To investigate the role of the opioidergic system. Opioid receptor antagonist, naloxone hydrochloride (5 mg/kg, i.p.), was administered to group eight or morphine (5 mg/kg) to group nine, 15 minutes before administering herbacetin (200 µg/kg) or morphine. The treatments were given through intraperitoneal injection. After 60 minutes, we injected 20 µL of 2.5% formalin (103999, purity 37%, Sigma-Aldrich, Merck Burlington, MA, USA) into the subplantar region of the right hind paw to induce pain. We measured the nociceptive response by recording the time spent by each mouse licking the formalin injection site. We recorded the licking times in two phases: the early (neurogenic) phase, which was 0-5 minutes after formalin injection, and the late (inflammatory) phase, which was 15-30 minutes after formalin injection. We calculated the percentage of licking inhibition (using the following formula: PIL = [(Licking time (control) – Licking time (treatment) / Licking time (control)] × 100[36].

Capsaicin-Induced Paw Licking Test

The aim of this experiment was to assess the impact of herbacetin on the vanilloid receptor, or TRPV1, and its antinociceptive properties[35]. The experiment involved six groups of mice (n=6 mice/group) that were administered either distilled water with 5% DMSO (group 1 - vehicle control), herbacetin at various doses (groups 2-5 at 50, 100, 150, and 200 µg/kg, respectively), or the TRPV1 receptor antagonist, capsazepine (group 6 at a dose of 0.17 mmol/kg) *via* intraperitoneal injection. After an hour, each mouse's right hind paw was injected with 20 µL (1.6 µmol/paw) of capsaicin (211275, Sigma Aldrich, Merck, Burlington, MA, USA) *via* the intraplantar route, and the nociceptive response was recorded by measuring the duration of time each mouse spent biting or licking the injection site for a period of 0 to 5 minutes.

Glutamate-Induced Paw Licking Test

To assess the antinociceptive effects of herbacetin on glutamatergic receptors, an experiment was conducted on five groups of mice, each comprising six mice. The first group received

5% DMSO intraperitoneally (i.p.) as vehicle control, while the remaining four groups were administered varying doses of herbacetin (50, 100, 150, and 200 µg/kg) i.p. After 60 minutes, 20 µL of glutamate (1446600, Sigma-Aldrich, Merck, Burlington, MA, USA) was injected into the ventral surface of the right hind paw of each animal. The mice were then observed for 15 minutes, and the duration each mouse spent licking and/or biting the glutamate injection site was recorded[37].

Proinflammatory Cytokines Measurements

Three hours after the formalin test, serum was collected to assess the IL-1β, TNF-α, IFN-γ, and NO serum using a two-site, sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (CUSABIO, Wuhan, Hubei, China).

Statistical Analysis

Data analysis was conducted using the Prism 5 software (GraphPad Software, Boston, MA, USA). The data are reported as the mean ± standard error of the mean (SEM). Differences between groups were assessed using one-way ANOVA, followed by Tukey's post-hoc test. Statistical significance was defined at $p<0.05$.

Results

Herbacetin Reduced Writhing Induced by Acetic Acid

Administering herbacetin intraperitoneally at doses of 100, 150, and 200 µg/kg resulted in a significant decrease ($p<0.001$) in the number of acetic acid-induced writhing episodes in mice that received treatment, as compared to the control group (Figure 1). Additionally, the effect of herbacetin was observed to be dependent on the dose ($p<0.05$). The group that was given the highest dose of herbacetin demonstrated a reduction of approximately 65% in the percentage of writhing inhibition in comparison to the control group ($p<0.001$). In contrast, the reduction in nociceptive behavior caused by the reference drug, ASA (100 mg/kg), was roughly 79% when compared to the control group (Figure 1).

Herbacetin Increased Latency Time in the Hot Plate Test

The administration of herbacetin at various doses, including 50, 100, 150, and 200 µg/kg, resulted in a significant ($p<0.001$) increase in the time taken by the animals to lick their posterior paw when placed on a hot plate (Figure 2). Additionally, the effect of herbacetin was observed to be dependent on the dose ($p<0.05$). The highest

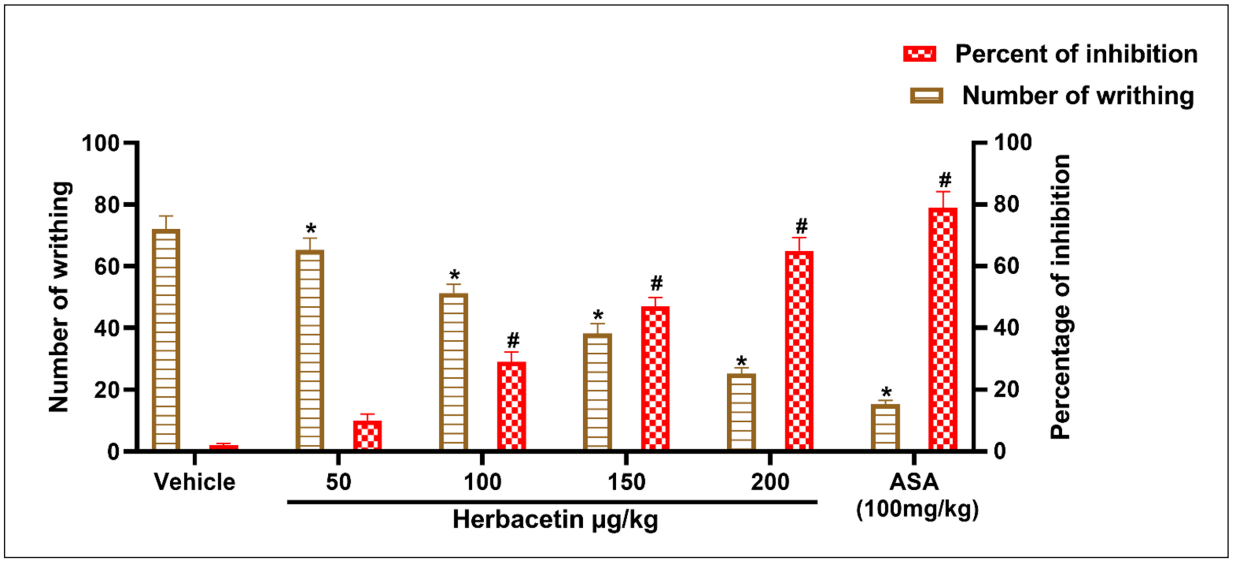

Figure 1. The impact of herbacetin on writhing induced by 0.6% acetic acid in mice. The experiment included six mice that received injections of either 5% DMSO (vehicle), herbacetin at different doses (50, 100, 150, 200 µg/kg i.p.), or acetylsalicylic acid (ASA) at a dose of 100 mg/kg. The mean values ± SEM were calculated, and the statistical analysis revealed that herbacetin caused a significant reduction in the number of writhing compared to the vehicle (*$p<0.001$). Similarly, the percentage of inhibition caused by herbacetin was also significantly different from the vehicle (#$p<0.001$). ANOVA was used for statistical analysis, followed by Tukey's post hoc test.

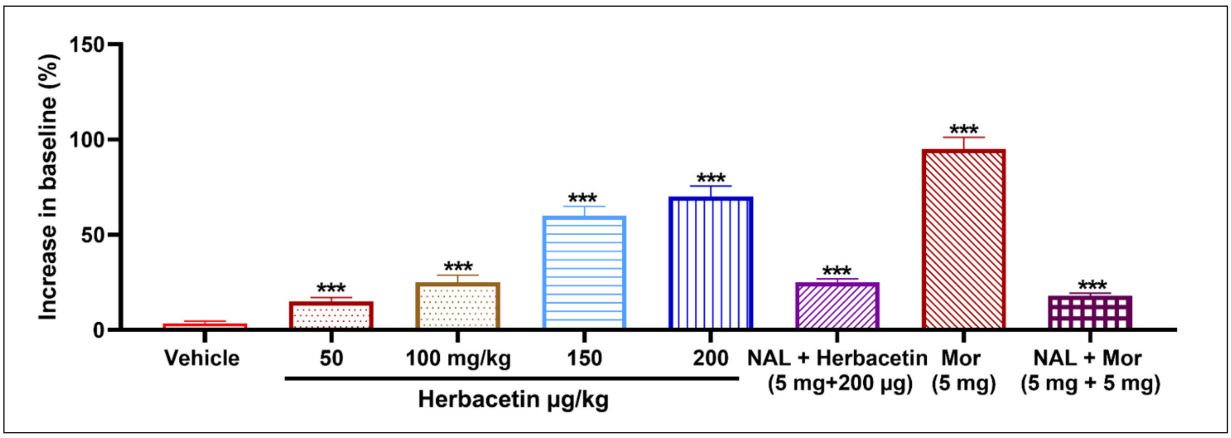

Figure 2. A group of six mice were subjected to the hot plate test to determine the effect of herbacetin. The mean values, along with the standard error of the mean (S.E.M.) were calculated. A significant difference was observed when compared to the vehicle (5% DMSO), as denoted by ***p<0.001. Additionally, a significant difference was noted compared to 200 µg/kg herbacetin or to 5mg morphine, which is indicated by ###p<0.001. The statistical analysis was carried out using ANOVA and Tukey's post hoc test. NAL refers to naloxone, and Mor refers to morphine.

dose of herbacetin (200 µg/kg) demonstrated the most noteworthy reduction in pain when compared to the control group. The herbacetin-treated group (200 µg/kg) displayed a 70% increase in baseline latency time, which was statistically significant (p<0.001). In comparison, morphine, which was utilized as a reference drug, caused a greater increase in latency time (95%).

To investigate the antinociceptive mechanism of herbacetin, we administered naloxone (an opioid antagonist) to mice 15 minutes prior to the administration of either 200 µg/kg of herbacetin or 5 mg/kg of morphine, followed by subjecting the mice to the hot plate test. The administration of naloxone significantly blocked the morphine-induced increase in latency time (p<0.001) compared to morphine treatment alone. Similarly, naloxone significantly blocked the increase in latency time induced by herbacetin (200 µg/kg) (p<0.001) compared to herbacetin treatment alone (Figure 2). However, some portion of the antinociceptive effect of herbacetin remained unaffected, indicating that herbacetin utilizes a different antinociceptive mechanism in addition to the opioidergic system.

Herbacetin Reduced Licking Time in Both the Early and Late Phases After Formalin

In this study, mice were administered different doses of herbacetin (100, 150, and 200 µg/kg) and were observed for reductions in licking times after formalin injection. The findings revealed that all doses of herbacetin led to significant reductions in both the early and late phases of licking times (Figure 3) in a similar pattern. Morphine, which was utilized as a reference drug, also resulted in a significant reduction in both phases. However, acetylsalicylic acid did not show any significant effect on the early phase of licking time (Figure 3A), but it did produce a significant reduction in the late phase (p<0.001, Figure 3B).

To investigate the involvement of the opioidergic system in the pain-relieving effects of herbacetin and morphine, mice were given naloxone 15 minutes prior to receiving either herbacetin (200 µg/kg) or morphine (5 mg/kg) and then subjected to the formalin-induced paw-licking test. The outcomes demonstrated that when naloxone was given before morphine, it significantly increased the licking time in both early and late phases compared to the group that received only morphine (p<0.001). In contrast, when naloxone was given before herbacetin, the licking times were significantly reduced in both early and late phases to the same degree (p<0.001), indicating that the antinociceptive effects of herbacetin are mediated by a mechanism of action other than the opioidergic system.

Licking Time was Reduced with Herbacetin Treatment After Capsaicin Injection

The aim of this experiment was to investigate the impact of herbacetin on the vanilloid system's

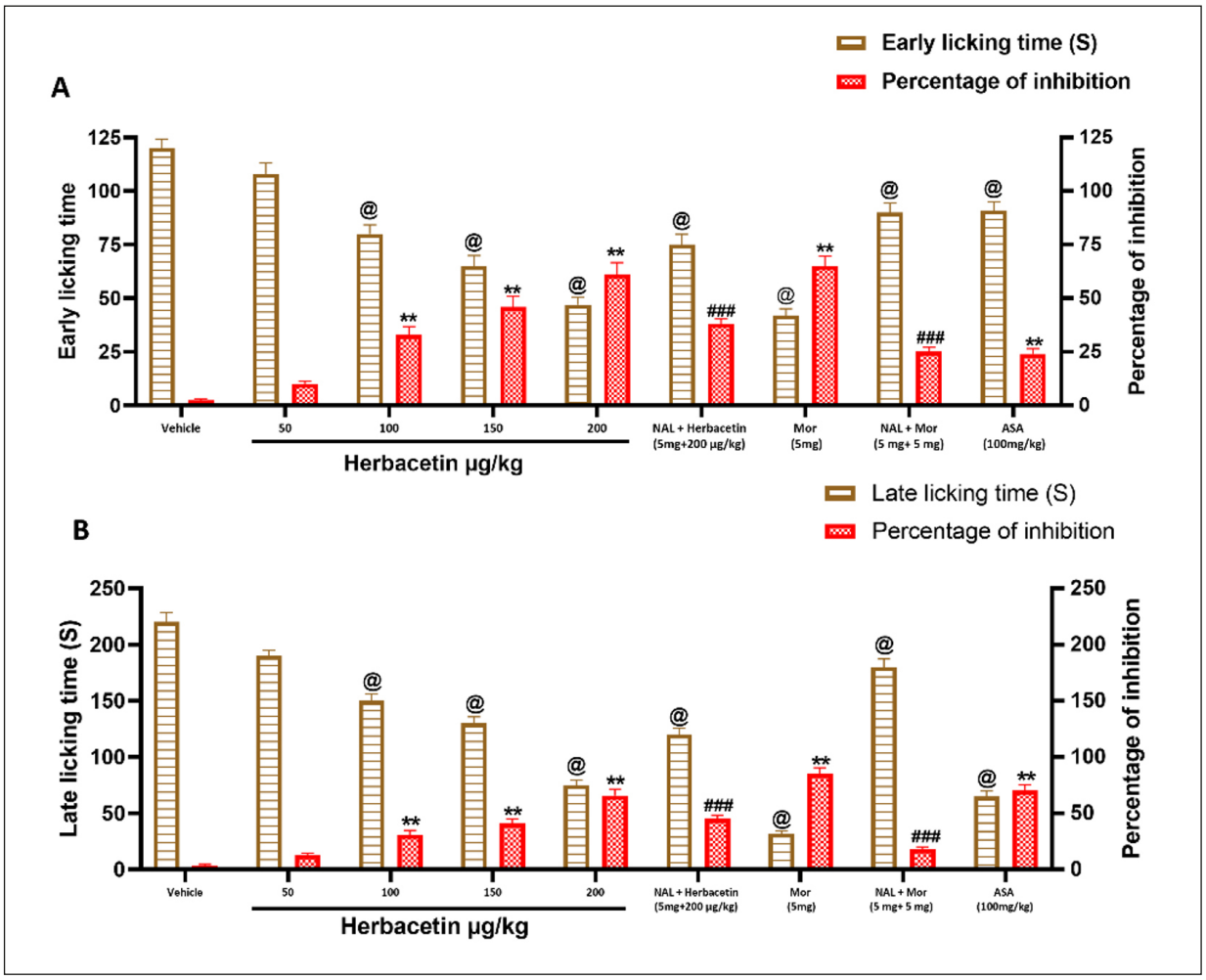

Figure 3. This figure describes an experiment that evaluated the effects of herbacetin on two different phases early (**A**) and late phase (**B**), of paw-licking behavior in mice that were induced with 2.5% formalin. The mice were given various injections, including 5% DMSO (vehicle), and different doses of herbacetin, morphine, or acetylsalicylic acid (ASA). In addition, naloxone was given to a group of mice before they were injected with herbacetin or morphine. The results are presented as mean values with standard error of the mean (SEM). The symbols $^{**}p<0.001$ and $@p<0.001$ indicate significant differences in the percentage of inhibition and licking time, respectively, compared to the vehicle group. The symbol $\#\#\#p<0.001$ indicates significant differences compared to the groups treated with herbacetin (200 µg/kg) or morphine (5 mg/kg, i.p.). Statistical analysis was performed using ANOVA and Tukey's post hoc test.

antinociceptive effects. To do so, mice were given different doses of herbacetin (50, 100, 150, and 200 µg/kg), and the licking time was recorded (Figure 4). The results revealed that all doses of herbacetin significantly ($p<0.01$) decreased the licking time in mice, and the effect was found to be dose-dependent. Additionally, when treated with capsazepine, a capsaicin antagonist, the licking time in mice was significantly reduced ($p<0.01$) with a percentage reduction of 75% (Figure 4), indicating that herbacetin exerts its antinociceptive effect, at least partially, through the vanilloid system.

Herbacetin Reduced Paw Licking Time After Glutamate Injection

Mice that were injected with glutamate and then administered herbacetin at doses of 100, 150, and 200 µg/kg exhibited a significant reduction ($p<0.001$) in paw licking time (Figure 5). Additionally, the effectiveness of herbacetin was found to be dose-dependent ($p<0.05$).

Proinflammatory Cytokines Levels

We measured four pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ, and NO) to study the anti-inflammatory and antinociceptive mechanism of

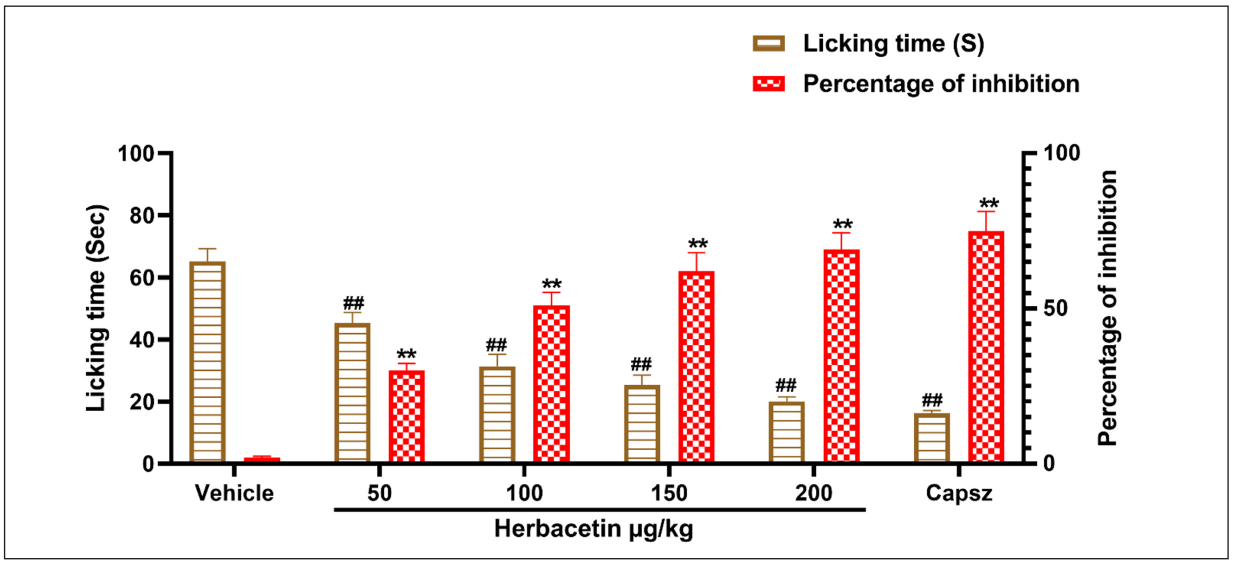

Figure 4. The effect of herbacetin on capsaicin-induced paw licking in mice. The effect was investigated by dividing the animals into five groups, each containing six male mice. The mice were injected with either 5% DMSO (vehicle), herbacetin at doses of 50, 100, 150, and 200 µg/kg (i.p.), or the capsaicin antagonist, capsazepine (Capsz, 0.17 mmol/kg, i.p.). After 60 minutes, the mice were challenged with capsaicin, and the time taken for paw licking was recorded. The results indicated that herbacetin caused a significant decrease in the percentage of inhibition compared to the control group, as indicated by $^{**}p<0.01$. Additionally, herbacetin resulted in a significant difference in licking time compared to the control group, denoted by $\#\#p<0.01$. The statistical analysis was carried out using ANOVA, followed by Tukey's post hoc test. The mean values along with the standard error of the mean (SEM) are reported.

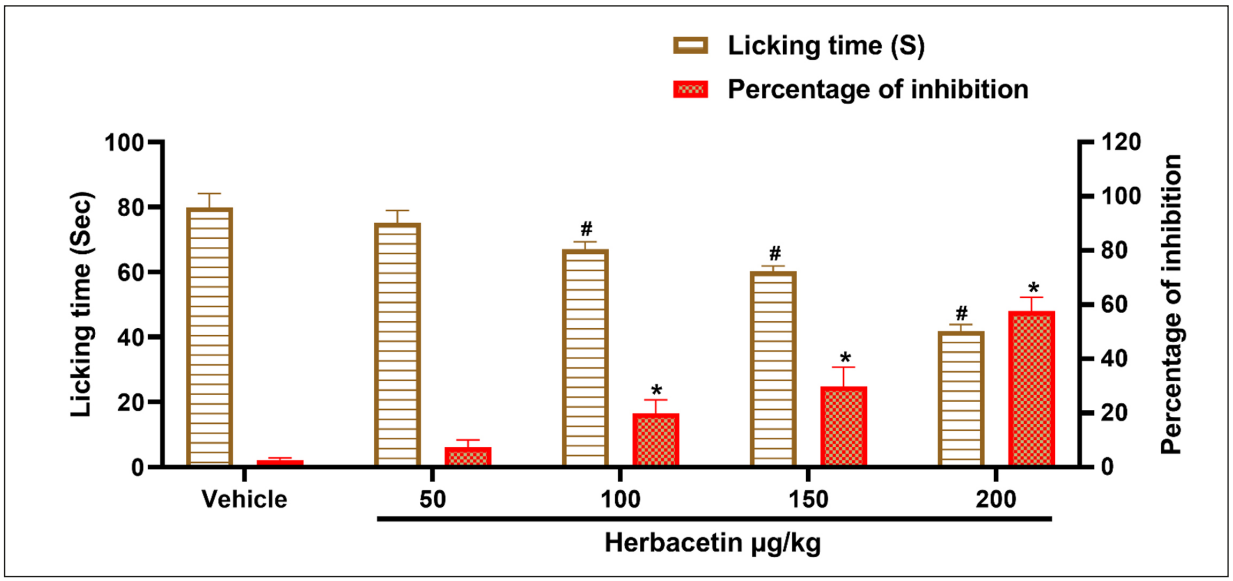

Figure 5. The effect of herbacetin on glutamate-induced paw licking in mice was investigated by dividing the animals into five groups, each containing six male mice. The mice were injected intraperitoneally with either 5% DMSO (vehicle) or herbacetin at doses of 50, 100, 150, and 200 µg/kg. After 60 minutes, 20 µL of glutamate was intraplantarly administered into the right hind paw, and the time taken for paw licking was recorded. The results indicated that herbacetin caused a significant decrease in the percentage of inhibition compared to the control group, as indicated by $^{*}p<0.001$. Additionally, herbacetin resulted in a significant difference in licking time compared to the control group, denoted by $\#p<0.001$. The statistical analysis was carried out using ANOVA, followed by Tukey's post hoc test. The mean values along with the standard error of the mean (SEM) are reported.

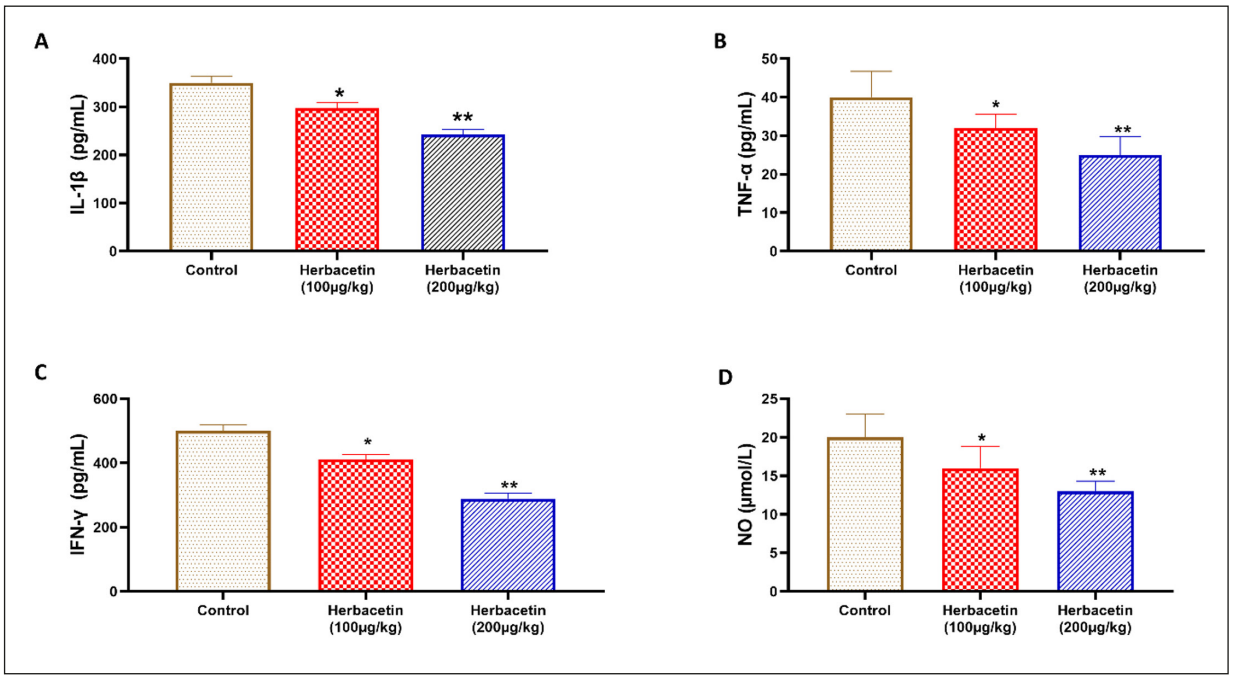

Figure 6. The effect of herbacetin on cytokines level. The results indicated that herbacetin caused a significant decrease in IL-1β (**A**), TNF-α (**B**), INF-γ (**C**), and NO (**D**), as indicated by *p<0.001. The statistical analysis was carried out using ANOVA, followed by Tukey's post hoc test. The mean values, along with the standard error of the mean (SEM), are reported.

herbacetin. As shown in Figure 6, the levels of IL-1β and NO were significantly inhibited by herbacetin -100 µg/kg and herbacetin -200 µg/kg (Figure 6). The inhibition of TNF-α and IFN-γ showed a dose-dependent relationship with herbacetin.

Discussion

According to the study's findings, it has been shown for the first time that herbacetin has antinociceptive effects. The results demonstrate that herbacetin is capable of inhibiting the nociceptive effects induced by acetic acid, a well-known pain inducer. The nociceptive effects of acetic acid are believed to be caused by the release of nitric oxide (NO) and inflammatory cytokines such as TNF-α, IL-1β, and IL-8 from peritoneal mast cells and macrophages, which in turn activate peripheral nociception receptors in the peritoneum[38,39]. Le Bars et al[40] reported that the sensation of nociceptive pain was quickly felt after the intraperitoneal (i.p.) administration of acetic acid. In this study, it was noted that the administration of acetic acid resulted in physical responses characterized by writhing. The reflex mechanism underlying this response involves the conversion of arachidonic acid into eicosanoids, which are essential pain mediators, by the enzymes lipoxygenases (LOX) and cyclooxygenases (COX)[41]. The release of prostaglandins and other mediators from the peritoneal cavity is stimulated by eicosanoids[42]. Furthermore, eicosanoids produced by COX and LOX increase pain sensitivity by sensitizing the peripheral pain neurons, resulting in hyperalgesia[43].

ASA and other NSAIDs have been found to inhibit prostaglandin synthesis by blocking COX activity, leading to a reduction in the body's response to pain[44]. The present study suggests that herbacetin may have inhibited acetic acid-induced nociception by suppressing peripheral COX and LOX levels. This, in turn, would have indirectly reduced the production of pain mediators, including prostaglandins, indicating that herbacetin has a peripheral antinociceptive effect. However, caution must be exercised since other non-analgesic agents, such as antihistamines, anticholinergic agents, and muscle relaxants, can lead to false-positive results in the acetic acid-induced writhing test[45]. Therefore, the study employed additional methods, such as the formalin and hot plate tests, to determine whether the antinociceptive effects of herbacetin are centrally or peripherally mediated.

The hot plate test is a method used to assess the supraspinal and spinal biological properties of new drugs, excluding inputs from peripheral nociception neurons[46]. An increase in the latency of mice experiencing discomfort on the hot plate by a drug or substance indicates centrally mediated activity, similar to that of opioids[47,48]. In this study, the results showed that herbacetin increased the latency of mice in experiencing discomfort on the hot plate, indicating the centrally mediated antinociceptive activity of herbacetin. Based on the results of the acetic acid-induced writhing test and the hot plate test, it is suggested that herbacetin may exert both central and peripheral antinociceptive activities.

The formalin-induced paw-licking test was performed to confirm the antinociceptive activity of herbacetin at both central and peripheral levels[49]. Our findings reveal that herbacetin effectively suppresses nociceptive responses in both phases, indicating its potential as a centrally-acting analgesic agent. This is significant because opioids, which are centrally-acting agents, are known to inhibit both phases, while drugs that act peripherally only inhibit the second phase (e.g., NSAIDs). Moreover, our study shows that pre-treatment with the non-selective opioid antagonist, naloxone, significantly reduced the antinociceptive effect of morphine but only partially inhibited the antinociceptive effect of herbacetin in the hot plate test and the early phase of formalin-induced nociception. These results suggest that, besides the opioidergic system, herbacetin may engage another mechanism to exert its antinociceptive action.

To investigate the potential mechanisms of action of herbacetin in modulating pain reception, we performed capsaicin-induced and glutamate-induced paw-licking tests. Capsaicin is known to induce the release of pro-inflammatory mediators from the periphery, including neuropeptides, neurokinins, nitric oxide, and excitatory amino acids such as glutamate and aspartate. Capsaicin can also transduce nociceptive pain signals from vanilloid receptors to the spinal cord[50]. Inflammatory mediators can activate and sensitize vanilloid receptors, creating a loop that amplifies the levels of inflammatory mediators and potentiates nociception[51].

According to our findings, herbacetin had a significant inhibitory effect on paw licking induced by capsaicin. This effect was comparable to the inhibitory effect produced by capsazepine, a well-known TRPV antagonist, as depicted in Figure 5. Our results suggest that herbacetin has the potential to interfere with the transmission of pain through vanilloid receptors and to block the release or activity of inflammatory agents caused by capsaicin. Additionally, our observation that herbacetin can alleviate the writhing and inflammatory responses in the second phase of the formalin test supports the idea that herbacetin can interfere with the activity of inflammatory mediators. The reduction in licking time observed with capsazepine in our experiments confirms the validity of the capsaicin-induced pain model and supports the effectiveness of our treatment with herbacetin.

To examine whether herbacetin can interfere with glutamate-mediated pain transmission, we conducted a glutamate-induced paw-licking test. Our results showed that mice exhibited licking behavior following glutamate injection, but this behavior was significantly reduced by different concentrations of herbacetin. This test is comparable to the late phase of the formalin test, which is typically inhibited by glutamate receptor antagonists like 2-Methyl-6-(phenylethynyl)pyridine (MPEP) or 7-hydroxyiminocyclopropan[b] chromen-1α-carboxylic acid ethyl ester (CPCCOE)[52,53]. Notably, the effect of herbacetin was restricted to the late phase of the formalin test and did not affect the neurogenic early phase.

Our study provides evidence that herbacetin can inhibit glutamate-induced pain responses, indicating that it can regulate pain transmission mediated by the glutamatergic system. This effect could be attributed to herbacetin's interaction with glutamate receptors or its ability to obstruct the release of NO, which is known to participate in pain signaling. Further investigation is required to fully understand the role of NO and its downstream signaling in the analgesic action of herbacetin. The mechanism of anti-inflammatory and antinociceptive effects are related to many processes including the cytokine and NO. Herbacetin inhibited (IL-1β, TNF-α, IFN-γ, and NO) in serum. Therefore, we deduced that herbacetin was a multi-target-directed drug with anti-inflammatory and antinociceptive effects.

Conclusions

Our findings reveal that herbacetin exerts antinociceptive effects through diverse physiological pathways in both the peripheral and central nervous systems. Specifically, herbacetin acts as an opioid receptor agonist and has the ability to inhibit the vanilloid and glutamatergic receptor systems.

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

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The necessary approvals for animal care and experimental procedures were obtained from the Animal Research Ethics Committee at Hashemite University (IRB number: HU 104/2022), and all procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Data Availability
Data are available upon reasonable request.

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Conflicts of Interest
The authors declare no conflicts of interest.

Informed Consent
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References

1) Walters ET, Williams ADCC. Evolution of mechanisms and behaviour important for pain. Philos Trans R Soc Lond Biol Sci 2019; 374: 20190275.
2) Tracey WD. Nociception. Curr Biol 2017; 27: R129-R133.
3) Walters ET. Nociceptive biology of molluscs and arthropods: Evolutionary clues about functions and mechanisms potentially related to pain. Front Physiol 2018; 9: 1049.
4) Vardeh D, Mannion RJ, Woolf CJ. Toward a Mechanism-Based Approach to Pain Diagnosis. J Pain 2016; 17: T50-T69.
5) Langley P, Müller-Schwefe G, Nicolaou A, Liedgens H, Pergolizzi J, Varrassi G. The impact of pain on labor force participation, absenteeism and presenteeism in the European Union. J Med Econ 2010; 13: 662-672.
6) Wang Y, Chen P, Tang Ch, Wang YLY, Zhang H. Antinociceptive and anti-inflammatory activities of extract and two isolated flavonoids of Carthamus tinctorius L. J Ethnopharmacol 2014; 2: 944-950.
7) Sneddon LU. Comparative physiology of nociception and pain. Physiology 2018; 33: 63-73.
8) Julius D, Basbaum AI. Molecular mechanisms of nociception. Nat 2001; 413: 203-210.
9) Ming-Tatt L, Khalivulla S, Akhtar M, Mohamad A, Perimal E, Khalid M, Akira A, Lajis N, Israf D, Sulaiman M. Antinociceptive Activity of a Synthetic Curcuminoid Analogue, 2,6-bis-(4-hydroxy-3-methoxybenzylidene)cyclohexanone, on Nociception-induced Models in Mice. Basic Clin Pharmacol Toxicol 2012; 110: 275-282.
10) Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. Front Mol Neurosci 2017; 10: 284.
11) Mohammad-Zadeh M, Azhdari-Zarmehri H, Mosavi H, Haghdoost-Yazdi F, Nazeri H, Shabani M. Modulation of Different Phases of Formalin Test by Force Swim Stress. Basic Clin Neurosci 2014; 5: 303-307.
12) Dehkordi SB, Sajedianfard J, Owji AA. The effect of intra-cerebroventricular injection of insulin on nociception of formalin test in non-diabetic and short-term diabetic rat models. Iran J Vet Res 2017; 18: 108-112.
13) Dehkordi BS, Sajedianfard J, Owji AA. The effect of intra-cerebroventricular injection of insulin on the levels of monoamines on the raphe magnus nucleus of non-diabetic and short-term diabetic rats in the formalin test. Iran J Basic Med Sci 2019; 22: 915-921.
14) Numazaki M, Tominaga M. Nociception and TRP channels. Curr Drug Targets CNS Neurol Disord 2004; 3: 479-485.
15) O'Neill J, Brock C, Olesen AE, Andresen T, Nilsson M, Dickenson AH. Unravelling the Mystery of Capsaicin: A Tool to Understand and Treat Pain. Pharmacol Rev 2012; 64: 939-971.
16) Neugebauer V. Metabotropic glutamate receptors - Important modulators of nociception and pain behavior. Pain 2002; 2: 1-8.
17) Toker G, Küpeli E, Memisoğlu M, Yesilada E. Flavonoids with antinociceptive and anti-inflammatory activities from the leaves of Tilia argentea (silver linden). J Ethnopharmacol 2004; 95: 393-397.
18) Küpeli E, Yesilada E. Flavonoids with anti-inflammatory and antinociceptive activity from Cistus laurifolius L. leaves through bioassay-guided procedures. J Ethnopharmacol 2007; 112: 524-530.

19) Chen H, Pu J, Liu D, Yu W, Shao Y, Yang G, Xiang Z, He N. Anti-Inflammatory and Antinociceptive Properties of Flavonoids from the Fruits of Black Mulberry (Morus nigra L.). PLoS One 2016; 11: e0153080.

20) Wei X, Zhao Z, Zhong R, Tan X. A comprehensive review of herbacetin: From chemistry to pharmacological activities. J Ethnopharmacol 2021; 279: 114356.

21) Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160: 1-40.

22) Koh JH, Johnson M, Dasari S, LeBrasseur N, Vuckovic I, Henderson G, Cooper S, Manjunatha Sh, Ruegsegger G, Shulman G, Lanza I, Nair K. TFAM Enhances Fat Oxidation and Attenuates High-Fat Diet-Induced Insulin Resistance in Skeletal Muscle. Diabetes 2019; 68: 1552-1564.

23) Negre-Salvayre A, Guerby P, Gayral S, Laffargue M, Salvayre R. Role of reactive oxygen species in atherosclerosis: Lessons from murine genetic models. Free Radic Biol Med 2020; 149: 8-22.

24) Cenini G, Lloret A, Cascella R. Oxidative Stress and Mitochondrial Damage in Neurodegenerative Diseases: From Molecular Mechanisms to Targeted Therapies. Oxid Med Cell Longev 2020; 2020: 1270256.

25) Qiao Y, Liu XB. In vitro Free Radical Scavenging and Protein Oxidation Inhibitory Effects of Herbacetin. Food Sci 2013; 34: 106-110.

26) Jeong HJ, Ryu Y, Park S, Kim J, Kwon H, Kim JH, Park KH, Rho M, Lee WS. Neuraminidase inhibitory activities of flavonols isolated from Rhodiola rosea roots and their in vitro anti-influenza viral activities. Bioorg Med Chem 2009; 17: 6816-6823.

27) Jo S, Kim S, Kim DY, Kim MS, Shin DH. Flavonoids with inhibitory activity against SARS-CoV-2 3CLpro. J Enzyme Inhib Med Chem 2020; 35: 1539-1544.

28) Hyuga S, Hyuga M, Yoshimura M, Amakura Y, Goda Y, Hanawa T. Herbacetin, a constituent of ephedrae herba, suppresses the HGF-induced motility of human breast cancer MDA-MB-231 cells by inhibiting c-met and akt phosphorylation. Planta Med 2013; 79: 1525-1530.

29) Veeramani C, Alsaif MA, Al-Numair KS. Herbacetin, a flaxseed flavonoid, ameliorates high percent dietary fat induced insulin resistance and lipid accumulation through the regulation of hepatic lipid metabolizing and lipid-regulating enzymes. Chem Biol Interact 2018; 288: 49-56.

30) Proença C, Oliveira A, Freitas M, Ribeiro D, Sousa JLC, Ramos MJ, Silva AMS, Fernandes PA, Fernandes E. Structural Specificity of Flavonoids in the Inhibition of Human Fructose 1,6-Bisphosphatase. J Nat Prod 2020; 83: 1541-1552.

31) Choe KI, Kwon JH, Park KH, Oh MH, Kim MH, Kim HH, Cho SH, Chung EK, Ha SY, Lee MW. The Antioxidant and Anti-inflammatory Effects of Phenolic Compounds Isolated from the Root of Rhodiola sachalinensis A. BOR Mol 2012; 17: 11484-11494.

32) Li L, Sapkota M, Kim SW, Soh Y. Herbacetin inhibits inducible nitric oxide synthase via JNK and nuclear factor-κB in LPS-stimulated RAW264.7 cells. Eur J Pharmacol 2015; 765: 115-123.

33) Animals NRC. Guide for the Care and Use of Laboratory Animals. Guid Care Use Lab Anim 2011.

34) Koster R, Anderson M, De BE. Acetic acid for analgesic screening. Fed Proc 1959; 18: 412-417.

35) Zakaria ZA, Jaios ES, Omar MH, Abd Rahman S, Hamid SS, Ching SM, The LK, Salleh MZ, Deny S, Taher M. Antinociception of petroleum ether fraction derived from crude methanol extract of Melastoma malabathricum leaves and its possible mechanisms of action in animal models. BMC Complement Altern Med 2016; 1: 488.

36) Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain 1992; 1: 5-17.

37) 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; 2: 219-228.

38) Larson AA, Kovacs KJ, Cooper JC, Kitto KF. Transient changes in the synthesis of nitric oxide result in long-term as well as short-term changes in acetic acid-induced writhing in mice. Pain 2000; 86: 103-111.

39) Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato AB, Poole S, Ferreira SH, Cunha FQ. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur J Pharmacol 2000; 387: 111-118.

40) Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev 2001; 4: 597-652.

41) Ricciotti E, Fitzgerald GA. Prostaglandins and Inflammation. Arterioscler Thromb Vasc Biol 2011; 31: 986-1000.

42) Jang Y, Kim M, Hwang SW. Molecular mechanisms underlying the actions of arachidonic acid-derived prostaglandins on peripheral nociception. J Neuroinflammation 2020; 17: 1-27.

43) Meek IL, van de Laar MAFJ, Vonkeman HE. Non-Steroidal Anti-Inflammatory Drugs: An Overview of Cardiovascular Risks. Pharm 2010; 3: 2146-2162.

44) Sani MHM, Zakaria ZA, Balan T, Teh LK, Salleh MZ. Antinociceptive activity of methanol extract of Muntingia calabura leaves and the mechanisms of action involved. Evidence-based Complement. Altern Med 2012; 2012: 890361.

45) Sani MHM, Zakaria ZA, Balan T, Teh LK, Salleh MZ. Antinociceptive activity of methanol extract of Muntingia calabura leaves and the mechanisms of action involved. J Evid Based Complementary Altern Med 2012; 2012: 890361.

46) Vidal-Torres A, Fernández-Pastor, B, Carceller A, Vela JM, Merlos M, Zamanillo D. Supraspinal and

peripheral, but not intrathecal, σ1r blockade by S1ra enhances morphine antinociception. Front Pharmacol 2019; 10: 422.

47) Lavich TR, Cordeiro RSB, Silva PMR, Martins MAA novel hot-plate test sensitive to hyperalgesic stimuli and non-opioid analgesics. Brazilian J Med Biol Res 2005; 38: 445-451.

48) Abdul Rahim MH, Zakaria ZA, Sani MHM, Omar MH, Yakob Y, Cheema MS, Ching SM, Ahmad Z, Abdul Kadir A. Methanolic extract of clinacanthus nutans exerts antinociceptive activity via the opioid/nitric oxide-mediated, but cGMP-independent, pathways. J Evid Based Complementary Altern Med 2016; 2016: 1494981.

49) Bjørklund G, Aaseth J, Doşa MD, Pivina L, Dadar M, Pen JJ, Chirumbolo S. Does diet play a role in reducing nociception related to inflammation and chronic pain? Nutrition 2019; 66: 153-165.

50) Caterina MJ, Julius D. The vanilloid receptor: A molecular gateway to the pain pathway. Annu Rev Neurosci 2001; 24: 487-517.

51) Cortright DW, Szallasi A. Biochemical pharmacology of the vanilloid receptor TRPV1: An update. Eur J Biochem 2004; 10: 1814-1819.

52) Bhave G, Karim F, Carlton SM, Gereau Iv RW. Peripheral group I metabotropic glutamate receptors modulate nociception in mice. Nat Neurosci 2001; 4: 417-423.

53) Gupta R, Gupta LK, Bhattacharya SK. Naloxone blocks the beneficial effects of aqueous extract of Murraya koenigii (L.) Spreng leaves in models of pain. Eur Rev Med Pharmacol Sci 2013; 17: 1748-1751.