Role of carbonic anhydrase in the anti-inflammatory mechanism of diosmin and hesperidin in rats

Running title: Role of carbonic anhydrase in the effect of diosmin and hesperidin

doi: 10.22201/fmvz.24486760e.2025.1381

Section: Original Research

Ady X. Muñiz-Alquicira[1]
0009-0005-3044-8433

Abel Zapata-Arenas[1]
0009-0005-4969-2647

Ruth Bustamante-García[1]
0009-0009-4687-5965

Atonatiu E. Gómez-Martínez[1]
0009-0005-6825-188X

Azucena I. Carballo-Villalobos[2]*
0000-0001-7104-8501

[1] Universidad Nacional Autónoma de México. Facultad de Química. Departamento de Biología. Ciudad de México, México.

[2] Universidad Nacional Autónoma de México. Facultad de Química. Departamento de Química Inorgánica y Nuclear. Ciudad de México, México.

*Corresponding author: azucenaicv@quimica.unam.mx

Dates:

Submitted: 2024-08-09
Accepted: 2024-09-27
Published: 2025-03-31

Cite this as:

Muñiz-Alquicira AX, Zapata-Arenas A, Bustamante-García R, Gómez-Martínez AE, Carballo-Villalobos AI. Role of carbonic anhydrase in the anti-inflammatory mechanism of diosmin and hesperidin in rats. Veterinaria Mexico OA. 2025;12.
doi: 10.22201/fmvz.24486760e.2025.1381.

Role of carbonic anhydrase in the anti-inflammatory mechanism of diosmin and hesperidin in rats

Abstract

Flavonoids such as diosmin and hesperidin produce antinociceptive and anti-inflammatory effects, potentially involving the inhibition of carbonic anhydrase (CA) as a mechanism of action. It is believed that CA inhibition helps reduce inflammation by improving symptoms such as pain, redness, swelling, warmth, and loss of mobility in the affected area. We showed that diosmin and hesperidin produced an anti-inflammatory effect involving inhibition of CA. The study was conducted on male Wistar rats by measuring the time spent licking and the number of shakings after the 1 % formalin was administered to the plantar area of the hind limb. Treatments were as follows: vehicle, diosmin, hesperidin, meloxicam, acetazolamide, sulfonamide, and combinations of inhibitors with the flavonoids. Subsequently, measurements in both hind limbs were carried out to evaluate the degree of inflammation. The results indicated that diosmin and hesperidin at 100 and 316.2 mg/kg decreased the time-spent licking and number of shakings in phase 2 of the 1 % formalin test, whereas when administered in combination with acetazolamide and sulfonamide at 100 mg/kg, the anti-inflammatory effect of the flavonoids was reversed. These findings suggest that CA's activity plays an important role in the antinociceptive and anti-inflammatory effects of the flavonoids diosmin and hesperidin.

Keywords: Diosmin; Hesperidin; Inflammatory pain; Formalin test; Carbonic anhydrase.

Study contribution

In this study, the influence of carbonic anhydrase on the mechanism of action of diosmin and hesperidin is evaluated for the first time in an *in vivo* model, since so far it has been demonstrated *in vitro* studies that both flavonoids have an inhibitory effect on this enzyme, however, it has not been shown whether these flavonoids mediate their anti-inflammatory effect through the inhibition of this enzyme in an experimental model; therefore, this work is a starting point to evaluate the participation of anhydrase through a pharmacological strategy initially. Accordingly, the natural-origin flavonoids diosmin and hesperidin were evaluated as their therapeutic potential for alleviating pain and inflammation, where we sought to elucidate the possible mechanism of action implicated in these flavonoids through the inhibition of carbonic anhydrase.

Introduction

Nowadays, pain and diseases associated with inflammation represent a serious health problem of growing incidence, both for the number of patients affected and the tremendous impact on the quality of life of those who experience them. So, the need to look for therapeutic alternatives has increased in recent years. Flavonoids are secondary metabolites from plants that have been studied more in pain and inflammation, so they have been presented as a good resource for new treatments. Despite the various investigations concerning the different uses of flavonoids, their activity in the central nervous system is not precisely known.

Hesperidin significantly inhibits the synthesis of prostaglandins *in vivo*[1], and this reduces the production of nitric oxide, tumor necrosis factor-alpha (TNF-α), lipopolysaccharide-induced interleukin (IL-12), and interferon-alpha in a dose-depend

manner, effects that could be associated with the mechanism of action through which hesperidin reduces inflammation and nociception in several experimental models.[2] The involved mechanism in the antinociceptive effect of hesperidin is also attributed to its antioxidant activity by inhibiting the production of reactive oxygen species (ROS), which is one of the triggers of inflammation and pain processes that are produced by cells related to the defense mechanisms of the organism, such as the polymorphonuclear neutrophils.[3]

ROS released during the inflammatory processes include the superoxide anion ($O_2^{\cdot-}$), the hydroxide radical (OH^-), hydrogen peroxide (H_2O_2), which can trigger a cascade of signals that ends with the production of pain mediators such as prostaglandins produced by cyclooxygenase-2 (COX-2), TNF-α, and IL-1β.[3] Furthermore, several studies have correlated the production of ROS with pain, evaluating the presence or decrease of such species compared to pain intensity. The findings show that as the amount of ROS decreases, the pain also decreases. On the contrary, the amount of these substances increases during painful processes.[4, 5]

The mechanisms responsible for other reported pharmacological effects of hesperidin, such as the anti-inflammatory, are also not clearly described, but it is known that it can reduce TNF-α, IL-1β, and intercellular adhesion molecule 1 levels, in addition to its ability to inhibit neutrophils that generate $O_2^{\cdot-}$ and glycosylation end products.[6] This suggests that this flavonoid can induce its antinociceptive effect through several mechanisms since some of these products are also related to nociception. On the other hand, diosmin has been explored as the main component of Daflon (containing 90 % diosmin and 10 % other flavonoids expressed as hesperidin), which is used in clinics to

improve microcirculation. However, diosmin is also reported to possess various pharmacological activities, including anti-inflammation, antioxidation, cancer protection, liver protection, neuroprotective, cardiovascular protection, renal protection, and retinal protection.[7] Despite this, no specific drug targets have been identified yet.

Regarding its neuroprotective effect, it has been reported that diosmin combined with hesperidin significantly improved mechanical and thermal hyperalgesia in the rat chronic constriction injury model. These effects can be blocked by dopamine like 2-receptor (D2) antagonists, gamma-aminobutyric acid subtype A receptor $GABA_A$, modulators, and opioids, but not by the serotonin receptor subtype 5 HT(1A) inhibitor. Consequently, diosmin alone may exhibit antihyperalgesic effects through D2 and opioid receptors, accompanied by reduced expression of pro-inflammatory cytokines (IL-1β, TNFα, and IL-6).[8] In another study, diosmin was shown to attenuate pain and inflammation by inhibiting spinal cord cytokines (IL-1β, TNFα, and IL-33/stimulation tumorigenicity 2 receptor (St2)) and activating glial cells.[9] It was also demonstrated to have antinociceptive effects in various models of inflammation in rats and mice by antagonizing transient receptor potential vanilloid 1 without producing side effects.[7]

Recent researchers have validated CAs (metalloenzymes that catalyze the rapid conversion of carbon dioxide and water to bicarbonate and protons) as new therapeutic targets for the design of new anti-glaucoma drugs, anticonvulsant, anti-cancer drugs, among others.[10] Among the different isoforms in the human body, hCA II and VII are highly expressed in the nervous system, both centrally and peripherally, and can be modulated to relieve pain and decrease inflammation. The physiologically dominant isoform is hCA II, expressed in different central nervous system cells, such as

oligodendrocytes and astrocytes. Moreover, high levels of hCA VII are observed in the cortex, hippocampus, and thalamus, making them promising targets for alleviating this condition.[11]

In addition, different *in vitro* inhibition assays of CA isoforms have shown that natural phenolic compounds, such as hesperidin and other flavonoids, cause their inhibition, leading to their involvement in the discovery and design of new drugs, as well as providing a possible molecular mechanism to attenuate pain and inflammation.[12]

In this study, the influence of carbonic anhydrase on the mechanism of action of diosmin and hesperidin is evaluated for the first time in an *in vivo* model, since so far it has been demonstrated *in vitro* studies that both flavonoids have an inhibitory effect on this enzyme, however, it has not been shown whether these flavonoids mediate their anti-inflammatory effect through the inhibition of this enzyme in an experimental model; therefore, this work is a starting point to evaluate the participation of anhydrase through a pharmacological strategy initially. Therefore, the natural-origin flavonoids diosmin and hesperidin were evaluated as therapeutic potentials for alleviating pain and inflammation, where we sought to elucidate the possible mechanism of action implicated in these flavonoids through the inhibition of CA.

Materials and methods

Ethical statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Comité Institucional de Ética, Facultad de Química, Universidad

Nacional Autónoma de México (protocol code: OFFICIO/FQ/CICUAL/482/22 on July 27th, 2022).

Experimental animals, accommodation, and feeding conditions

Male Wistar rats weighing between 180 and 250 g were used in sixteen experimental groups, each consisting of six animals (N = 96). All experiments were conducted under controlled environmental conditions; the rooms were kept under a 12/12-hour dark-light cycle and included extraction and injection of air with at least 16 changes per hour; the temperature and relative humidity of the environment in the procedure room were maintained at 22 ± 2 °C and between 40 and 70 %, respectively. All the animals were provided with food and water *ad libitum*.

Drugs and reagents

The products used in this project were the following: Fluka® analytical diosmin with batch: SLBC3987V, Sigma-Aldrich® hesperidin with batch: SLBT3541, Sigma-Aldrich® acetazolamide with batch: BCCC4390, Sigma-Aldrich® sulfonamide with batch: 0000120872, Sigma-Aldrich® Tween 80 vehicle with batch: 9005656 prepared at 1 %; and as the active ingredient, meloxicam in its commercial presentation, Flaxol MX veterinary injectable solution of Biochem® with batch: ASD4887. For the 1 % formalin preparation, a 1.25 mL aliquot of the 40 % concentrate was taken and made up to 50 mL in a flask.

Experimental design

The oral route (PO) of administration was selected as the suggested route for the clinical use of flavonoids. Doses were based on the reported literature from studies with diosmin (D) and hesperidin (H),[8, 13] as well as for acetazolamide (A) and sulfonamide (S).[14, 15] Once the antinociceptive and anti-inflammatory effects of both flavonoids were evaluated at doses of 100 and 316.2 mg/kg, the dose of 100 mg/kg was chosen due to its significant response concerning the vehicle and the reference drug and without any significant difference in comparison to the dose of 316.2 mg/kg.

Evaluation of the involvement of carbonic anhydrase

To evaluate the influence of CA on the possible mechanism of action of flavonoids, the doses were tested in combination with the inhibitors A and S (groups D/A, H/A, D/S, and H/S, all at 100 mg/kg).

Treatments

The volume for the treatments' administration that each rat received was 0.1 mL/100 g body weight. The vehicle consisted of 1 % Tween 80. For the preparation of the doses of flavonoids, or drugs A and S, the necessary amount of each was weighed, and a suspension was made using 1 % Tween 80. Doses of 100 and 316.2 mg/kg, PO, were used to administer both flavonoids. Meloxicam, the antinociceptive reference drug, was used at a dose of 10 mg/kg PO. The drugs A and S were prepared for a dose of 100 mg/kg and then intraperitoneally administered. Flavonoids were given 30 minutes before the induction of nociception, and drugs A and S were administered 10 minutes before.

Evaluation of 1 % formalin-induced biphasic response

The rats were evaluated by placing them in methacrylate cylinders with a height of 25 cm and an area of 706.86 cm^2. For adaptation, each animal remained for at least 15 minutes in the methacrylate cylinder before the start of the test. Then, rats were injected into the sole of the right hind paw with 2 µL of 1 % diluted formalin with a 30-gauge needle and instantly returned to the cylinders. The nociceptive behavior was observed immediately, during the first five minutes (0–5 min), and then 20 minutes after the formalin administration for another five minutes (20–25 min). The nociceptive behavior of the paw was quantified as the number of shakings and the time the animal spent licking the paw during the two phases. At the end of the evaluations, euthanasia in a CO_2 chamber was performed. Immediately, the corresponding measurements of the plantar diameter were made in both hind limbs to quantify the degree of inflammation.

Evaluation by the thread test

For the measurement of inflammation, the paw circumference measurement method was used.[16] In this test, the width of the healthy limb and that with edema was measured to determine the degree of inflammation of the injected limb through the difference between both circumferences.

The anti-inflammatory activity was calculated by using the following relationship:

$$\%\ antiinflammatory\ effect = \frac{T - T_0}{T} x100$$

Where: T, thickness of paw in the control group; T_0, thickness of paw edema in the test compound-treated group.

Ethanol-induced gastric ulcer assessment

This test was performed to evaluate the gastric protection capacity of the flavonoids diosmin and hesperidin through the employed administration route, and in comparison with meloxicam as the reference drug, which produces gastrointestinal adverse effects.[17] The degree of gastric damage was assessed in groups with diosmin 100 mg/kg, hesperidin 100 mg/kg, and meloxicam 10 mg/kg. The gastric ulcer was induced with absolute ethanol in rats.[18] Thirty minutes after the treatment with the vehicle, flavonoids (100 mg/kg), and meloxicam (10 mg/kg), rats were given 1 mL of absolute ethanol, PO One hour later, rats were sacrificed in a CO_2 chamber to dissect their stomachs, which were subsequently filled with 10 mL of 10 % formaldehyde to be fixed. After 10 minutes, the greater curvature of the stomach was cut to expose the inner walls. Gastric damage was identified through a mucosal lesion with elongated bands of hemorrhagic lesions, which were captured and measured in millimeters with the ImageJ image processing program. The following formulas were used to obtain the results:

$$Ulceration\ Index\ (UI) = \frac{Ulceration\ area}{Total\ stomach\ area}$$

$$Gastroprotection\ (\%) = \frac{(Mean\ UI\ of\ control\ group - UI\ of\ treatment)}{(Mean\ UI\ of\ control\ group)}$$

Histopathological evaluation

Stomach samples were stored in the fixative solution (10 % buffered formalin), next, the tissues were processed (dehydration, clarification and paraffin impregnation), and cut into 3 µm thickness. Staining was carried out using hematoxylin and eosin. The analysis will be carried out by observing morphological changes of the stomachs.

Statistical analysis

For the 1 % formalin test, data are presented as the mean ± the standard error of the mean (SEM) in the time courses of the time-spent licking and the number of shakings. A two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, was used to compare the evaluated parameters between the groups. Differences were considered significant at $P \leq 0.05$. To measure the diameter of swollen extremities and gastric damage, a one-way analysis (ANOVA), followed by the post-hoc Tukey's test, was used. Differences were considered significant at $P \leq 0.05$. Data analysis was performed with the Prism GraphPad® version 5.01 program.

Results

Formalin test

The formalin-induced nociception produced the typical shaking and licking behavior, characterized by brief and rapid withdrawal or the injected paw flexion.[19] Graphing the time spent licking and the number of shakings based on the distinctive phases of this pain test,[20] the nociceptive behavior was evaluated in the control and treatment groups. Conventionally, the neurogenic phase is the result of central sensitization of dorsal horn

neurons and activity of C and A6 fibers, while the inflammatory phase is due to the release of inflammatory molecules and sensitization of the spinal cord, as well as decreased activity of C fibers.[21]

Analyzing the graph in **Figure 1A**, we found that in both phases, the flavonoids decreased the time spent licking and the number of shakings compared to the vehicle (control), demonstrating their analgesic-anti-inflammatory effect. A greater effect was observed in phase 1 on time-spent licking for diosmin at both doses relative to meloxicam, with a significant difference of $P < 0.05$, while hesperidin and meloxicam had the same effect level. Regarding the number of shakings, in phase 1, a greater antinociceptive response was obtained from both flavonoids in their two doses compared to meloxicam, with a difference of $P < 0.01$. Meloxicam did not show an attenuating effect on the number of shakings at this point, which can be attributed to the fact that this drug is a selective inhibitor of COX-2,[22] and since in the chronic state of inflammatory pain, COX-2 is the predominant isoenzyme at the site of inflammation and in the spinal cord, which occurs in phase 2.[23]

Additionally, in this inflammatory phase, the effect could be seen in the decreased number of shakings, having the same effect as the flavonoids (panel B in **Figure 1**). In phase 2 of both assessed behaviors, it was observed that flavonoids produced the same effect as achieved by meloxicam, which was significant for vehicle $P < 0.001$. Additionally, no significant difference was found between 100 mg/kg and 316.2 mg/kg in the diosmin and hesperidin evaluations.

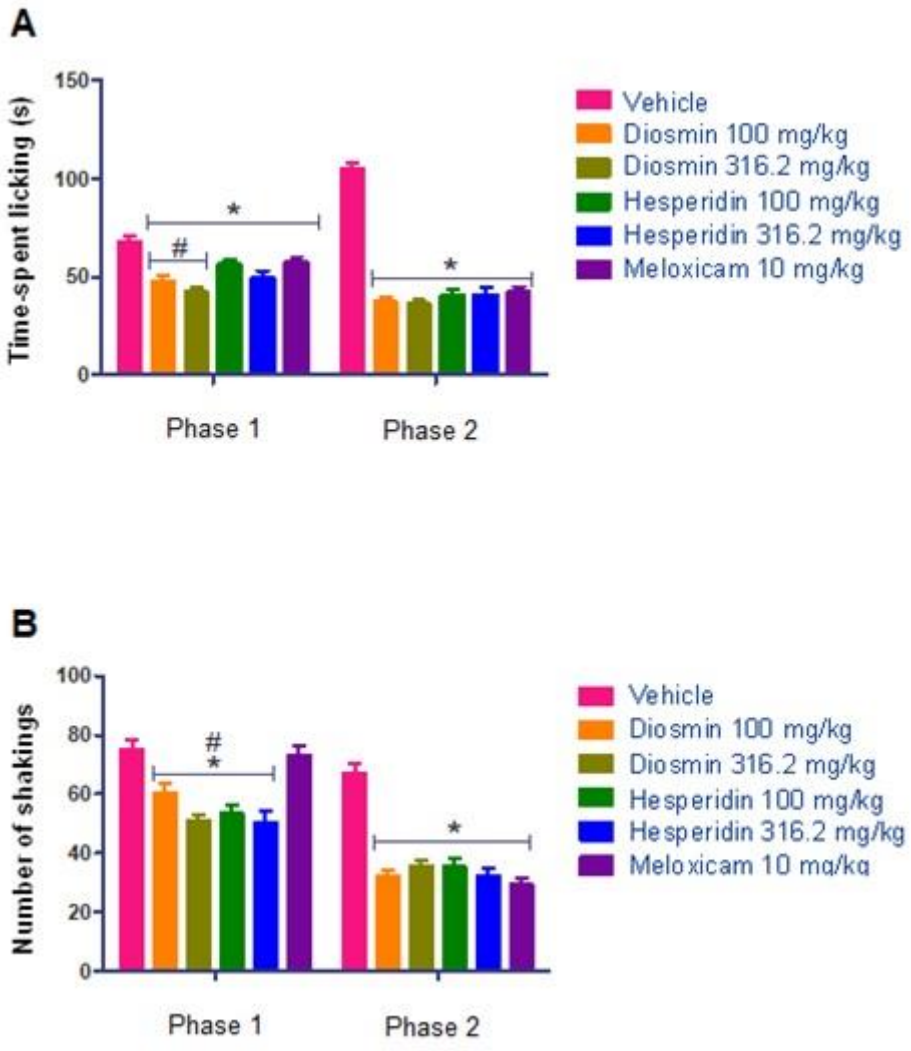

Figure 1. Evaluation of the anti-inflammatory effect of the flavonoids diosmin and hesperidin in the 1 % formalin test. In panel (A), each bar represents the mean ± SEM of the time spent licking (s) in two intervals, phase 1 (neurogenic) and phase 2 (inflammatory). Animals in the groups were administered diosmin and hesperidin at 100 mg/kg and 316.2 mg/kg, and meloxicam at 10 mg/kg. In panel (B), each bar represents the mean ± SEM of the number of shakings in phases 1 and 2 for the same treatments. In both panels, a two-way analysis of variance was performed: *$P < 0.05$ *vs*. vehicle, and $^{\#}P < 0.05$ *vs*. meloxicam. All groups consisted of at least six animals.

Standard Error of the Mean (SEM)

In **Figure 2**, both flavonoids at 100 mg/kg were compared against the A and S inhibitors at the same dose. Likewise, different combinations among these groups were also compared to demonstrate the participation of CA in the antinociceptive effect of diosmin and hesperidin. As shown in panels A and B of **Figure 3**, there was no therapeutic effect of A and S inhibitors when administered individually, as no significant difference was observed from the vehicle in both phases of the test. In contrast, when administered in combination with flavonoids, it was observed that the time-spent licking and the number of shakings increased significantly ($P < 0.001$) concerning the diosmin and hesperidin groups administered individually, thus decreasing the anti-inflammatory effect caused by the flavonoids and suggesting that both A and S somehow inhibit the therapeutic effect of diosmin and hesperidin.

Regarding phase 1, only diosmin and hesperidin showed a difference versus control in both behaviors (time-spent licking, $P < 0.01$, and number of shakings, $P < 0.05$) (**Figures 2A** and **2B**). In addition, there was no decrease in the measurement of behaviors with individual administration of A and S in this phase.

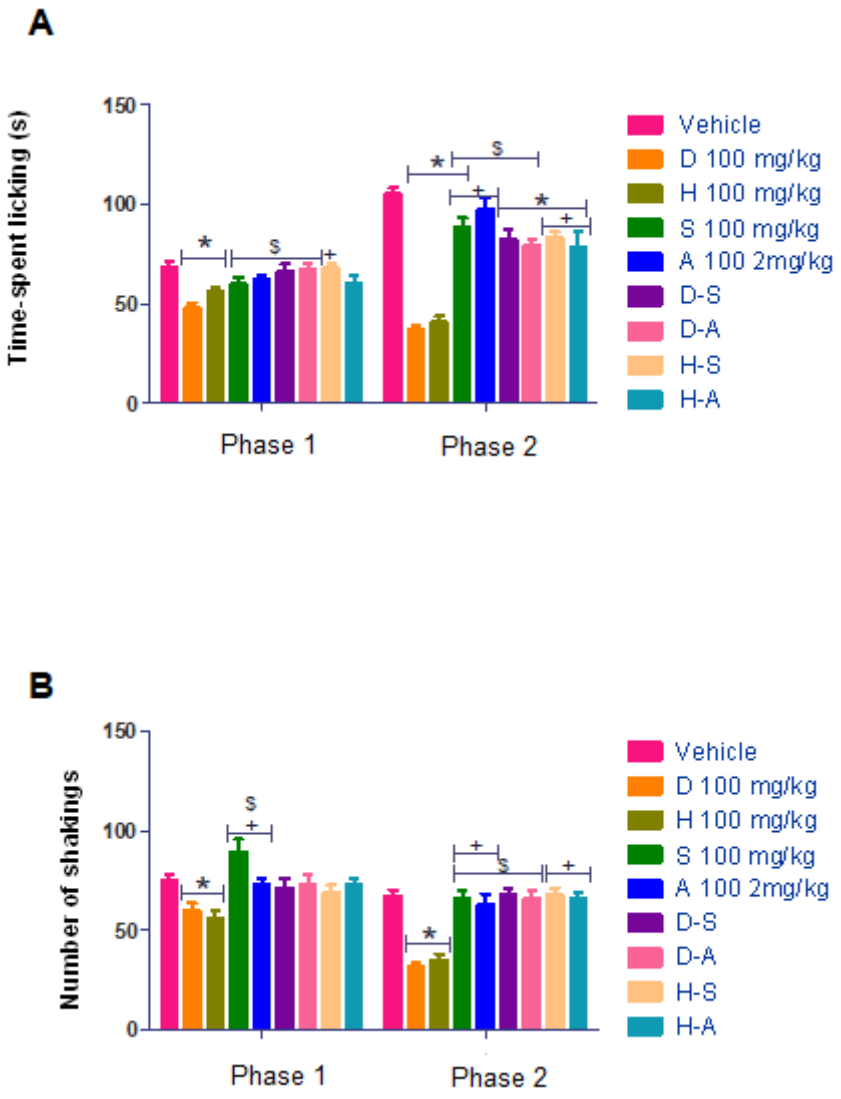

Figure 2. Evaluation of the anti-inflammatory effect of the flavonoids diosmin and hesperidin in the 1 % formalin test. In panel (A), each bar represents the mean ± SEM of the time spent licking (s) in two intervals, phase 1 (neurogenic) and phase 2 (inflammatory). Animals in the groups were administered with the vehicle, (D) diosmin and (H) hesperidin at 100 mg/kg, (A) acetazolamide and (S) sulfonamide at 100 mg/kg, and the different combinations with flavonoids (D-S, D-A, H-S, H-A). In panel (B), each bar represents the mean ± SEM of the number of shakings for phases 1 and 2 for the

same treatments. For both panels, a two-way analysis of variance was performed where *P ≤ 0.05 *vs*. vehicle, $^{+}$P ≤ 0.05 *vs*. hesperidin, and $^{\$}$P ≤ 0.05 *vs*. diosmin. All groups consisted of at least six animals.

Thread test

The edema was measured to determine the degree of inflammation of the injected limb. **Figure 3A** shows the anti-inflammatory effect of diosmin, hesperidin, and meloxicam compared to vehicle (P < 0.0001). In the same way, as in the formalin test, no significant differences were observed among the evaluated doses of flavonoids.

When diosmin and hesperidin were individually administered, they produced a high anti-inflammatory effect; however, combined with the A and S inhibitors, their effect was significantly decreased (panel B in **Figure 3**).

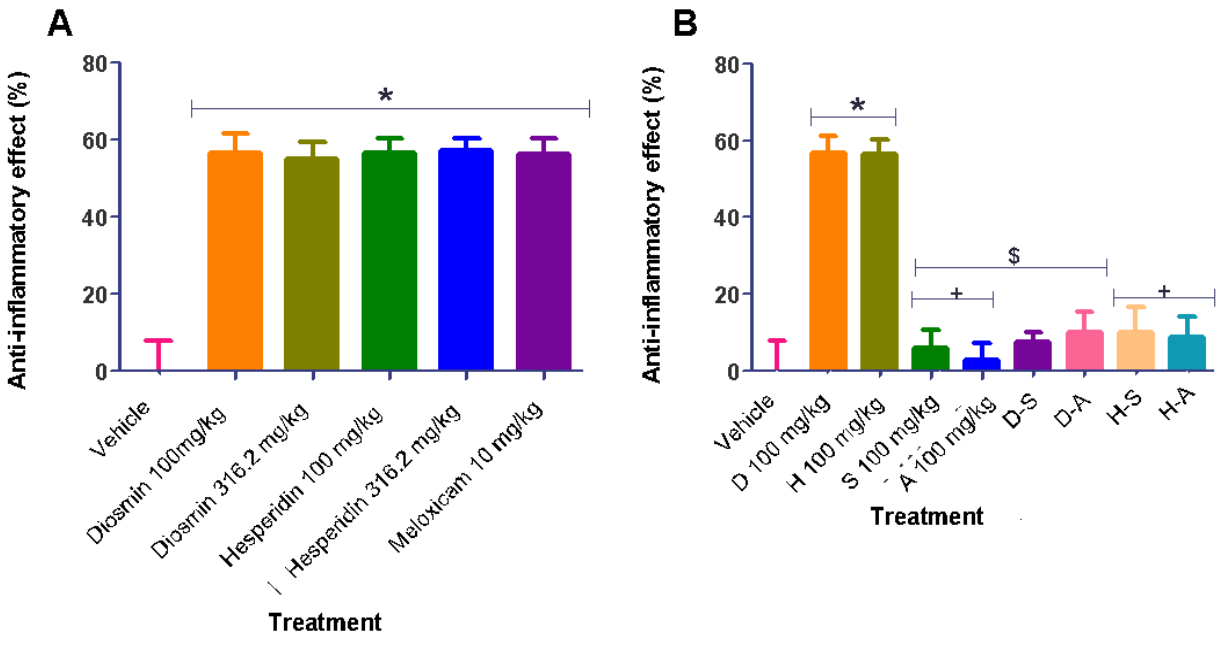

Figure 3. Evaluation of the anti-inflammatory effect of the flavonoids diosmin and hesperidin in the thread test. In panel (A), each bar represents the groups administered with diosmin and hesperidin at 100 and 316.2 mg/kg and meloxicam at 10 mg/kg. In panel (B), the groups administered with the vehicle, (D) diosmin and (H) hesperidin at 100 mg/kg, acetazolamide (A) and sulfonamide (S) at 100 mg/kg, and the different combinations among flavonoids and inhibitors (D-A, D-S, H-A, and H-S) are shown. For both panels, each bar represents the mean ± SEM of the percentage of anti-inflammation in at least six animals. A one-way analysis of variance followed by a Tukey's test was performed: *P ≤ 0.05 *vs.* vehicle, $^{+}$P ≤ 0.05 *vs.* hesperidin, and $^{\$}$P ≤ 0.05 *vs.* diosmin.

Gastroprotective effect of diosmin and hesperidin

We evaluated a possible adverse effect of treatments using the ethanol-induced gastric ulcer test. The diosmin and hesperidin groups showed a significant difference (P < 0.0001) regarding the percentage of gastroprotection in comparison with the reference drug meloxicam (**Figure 4A**). In panel B in **Figure 5**, the ulceration index (UI) or percentage of existing damage is shown, where a significant difference of P < 0.0001 with respect to the vehicle was observed in the diosmin, hesperidin, and meloxicam groups. A remarkable difference of P < 0.0001 against meloxicam was obtained with the flavonoids, indicating that both provide significant protection of the gastric mucosa compared to the vehicle and the drug meloxicam.

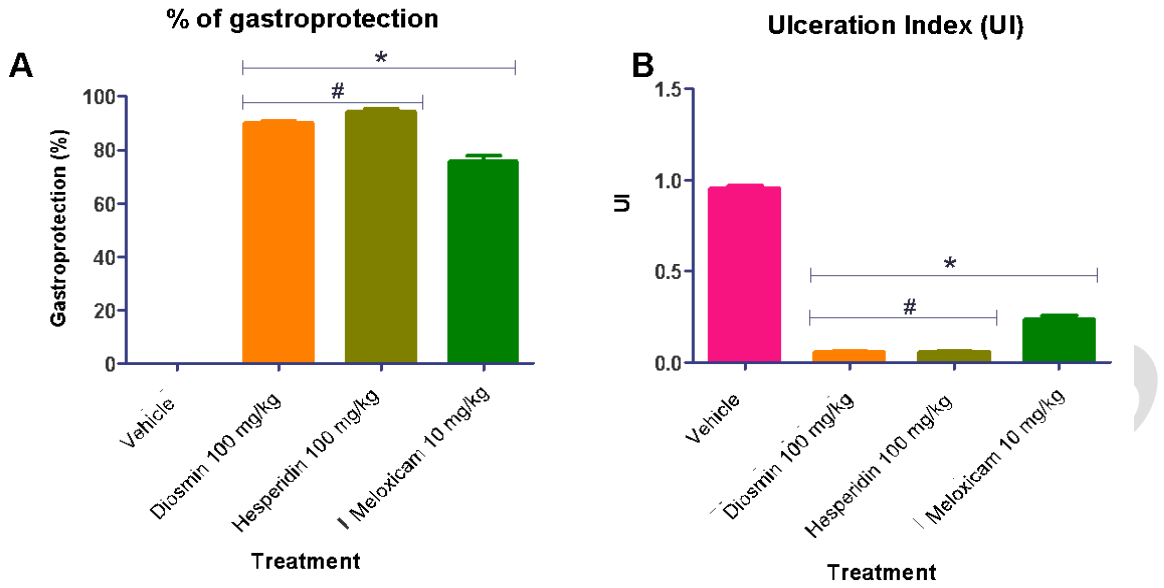

Figure 4. Gastroprotective effect of diosmin, hesperidin, and meloxicam on gastric lesions induced by absolute ethanol in Wistar rats. In panel (A), each bar represents the mean ± SEM of the percentage of gastroprotection in at least six animals. In panel (B), each bar represents the ulceration index (UI). For both panels, a one-way analysis of variance followed by a Tukey's test was performed: *P ≤ 0.05 *vs*. vehicle, and $^{\#}$P ≤ 0.05 *vs.* meloxicam.

Histopathological changes in gastric tissue

On the histopathological assessment, gastric tissue damage in rats receiving absolute ethanol was observed in the glandular section, an extensive area of the lamina propria with abundant erythrocytes outside the vascular bed, dissecting the glands. The submucosal fibers are separated by oedema, expanding the submucosa (diffuse mild gastric hemorrhage) (**Figure 5A** and **5E**). The group administered with diosmin 100 mg/kg shows a focus of the glandular section with accumulated erythrocytes in the middle and apical part of the mucosa (mild focal gastric congestion) (**Figure 5B** and **5F**).

Hesperidin (100 mg/kg) prevented the development of damage, as there were no obvious pathological changes (**Figure 5C** and **5G**). Meloxicam (10 mg/kg) produced a significant area of lesion in the glandular section, with accumulation of erythrocytes in the middle and apical part of the mucosa. The submucosal fibers are separated by oedema, expanding the submucosa (mild focal gastric congestion) (**Figure 5D** and **5H**).

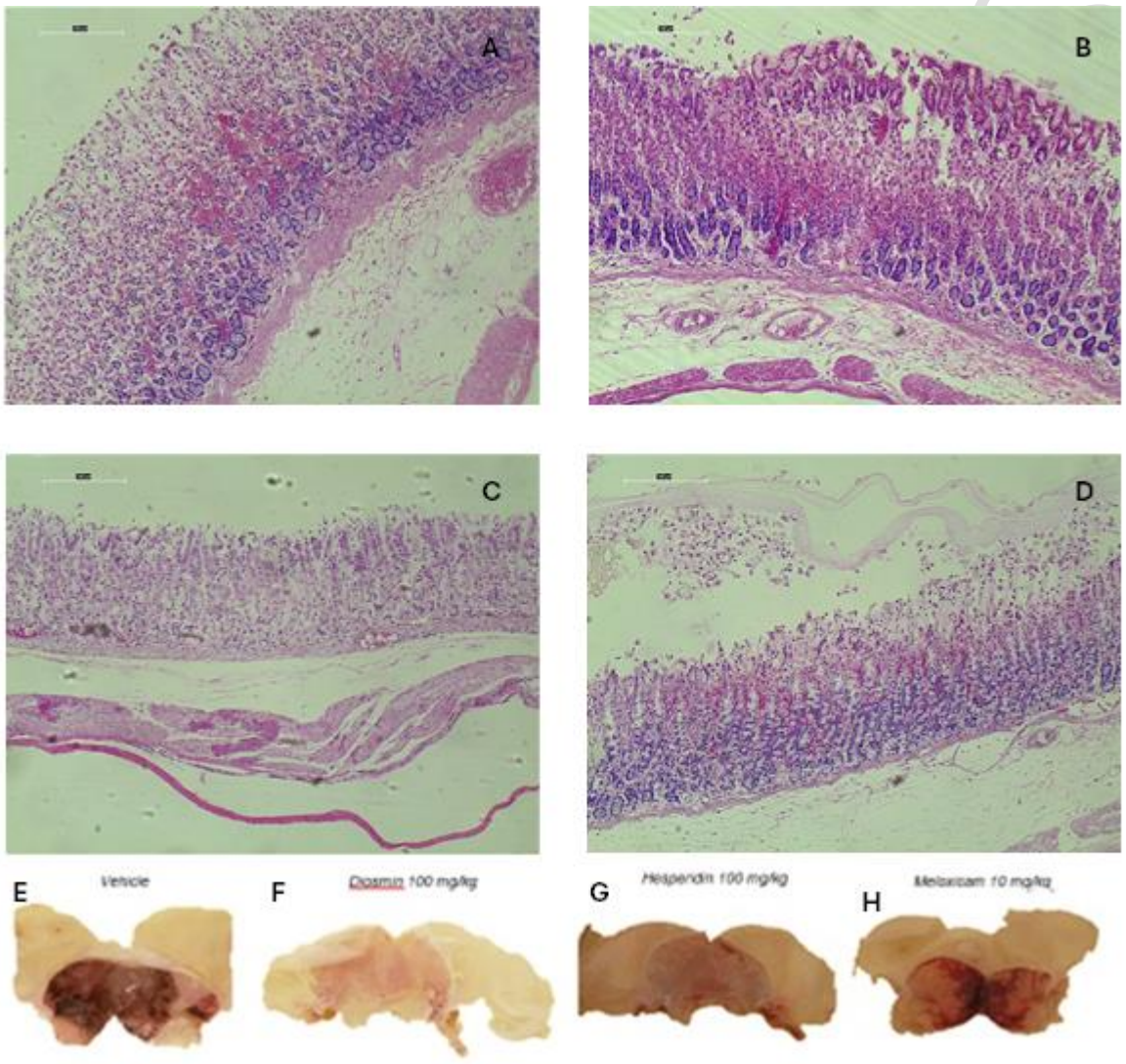

Figure 5. Representative photomicrographs with morphological changes of gastric lesions in rats. Panel A) (absolute alcohol, vehicle) mild diffuse gastric hemorrhage. B) Diosmin 100 mg/kg, mild focal gastric congestion. C) Hesperidin 100 mg/kg, no apparent

changes. and D) Meloxicam 10 mg/kg, mild focal gastric congestion. Below are representative photographs of gastric lesions in E) absolute alcohol, vehicle, F) diosmin, G) hesperidin and H) meloxicam.

Discussion

In the present study, the involvement of CA in the antinociceptive effect of the flavonoids diosmin and hesperidin was evaluated in acute and tonic pain induced with 1 % formalin in Wistar rats. It is reported in the literature that different flavonoids, including diosmin and hesperidin, cause a therapeutic effect,[24] in doses ranging from 5 mg/kg to 400 mg/kg without producing toxic effects in experimental models with mice and rats.[25, 26] For people, the dietary intake of these flavonoids in Western countries is estimated to be between 193 and 562 mg, which corresponds to 2.8 to 8 mg/kg of body weight for a 70 kg person.[27] In this study, we used doses of 100 and 316.2 mg/kg. Nonetheless, there was no significant difference among these two groups, so it was decided to use the lower dose of 100 mg/kg for the subsequent experiments.

The response of the flavonoids was compared to the reference drug, meloxicam. Analyzing the results in phase 1 of the formalin test, we noticed that the effect of the flavonoids diosmin and hesperidin on both assessed behaviors was greater than that of meloxicam. When phase 2 was reached, the effect of meloxicam was equal to that of flavonoids, since there were no significant differences among these groups. Meloxicam is a non-steroidal anti-inflammatory drug considered an inhibitor of COX-1 and COX-2 with greater selectivity for COX-2, COX-2 is typically absent in cells but is rapidly expressed in response to stimuli such as lipopolysaccharides or pro-inflammatory

cytokines, regulating the production of prostanoids involved in inflammatory and non-inflammatory processes.[28]

Even so, their mechanism of action and the involvement of CA have not been elucidated yet. CAs are zinc-containing metalloenzymes that catalyze the hydration and dehydration reactions of carbon dioxide (CO_2) and carbonic acid (H_2CO_3), respectively. The hydration reaction generates bicarbonate ions (HCO_3^-) and hydrogen ions (H^+) from CO_2, while the dehydration reaction generates CO_2 from H_2CO_3.[29] Margheri et al.[30] found that some CA isoforms (CAIV, CAIX, and CAXII) were overexpressed in the presence of inflammation, so they were considered therapeutic targets for this condition. Likewise, the CAII and CAVII isoforms were identified as therapeutic targets for neuropathic pain since they are found at relatively high levels in the cortex, hippocampus, and thalamus.[31]

In our study, the administration of the flavonoids diosmin and hesperidin resulted in decreased time-spent licking and shaking behaviors, as well as diminished limb inflammation, in comparison to the vehicle, acetazolamide, sulfonamide, and their respective flavonoid combinations. In phase 1, or “neurogenic”, the activation of Aδ fibers and C fibers occurs during the first minutes of the evaluation, resulting in the generation of action potentials that induce, in turn, the release of neurotransmitters in the dorsal horn of the spinal cord, causing the transmission and perception of pain. At this point, the pH of the medium remains balanced; however, as the minutes pass, the damage progresses and phase 2, or “inflammatory” is reached. In this phase, a cascade of inflammatory substances sensitizing or exciting nociceptors is triggered. These include K^+ and H^+ ions, serotonin, bradykinin, histamine, prostaglandins, leukotrienes, and substance P.[19, 20]

It has been reported that in inflamed tissues, there was a high concentration of hydrogen ions, indicating that the pH became more acidic as the intensity of the inflammatory reaction increased; pathophysiologically relevant pH values (6.1-6.9 threshold) produced a selective non-adapting excitation of nociceptors and a significant sensitization to mechanical stimulation. Thus, it has been proposed that local acidosis plays a major role in cutaneous pain and hyperalgesia, where the hydrogen ions selectively excite “polymodal” and “high-threshold mechanosensitive” C-fibers.[32] In addition, since CAs are overexpressed in inflammatory conditions, we can suggest that these enzymes contribute to acidifying the environment by increasing pCO_2 and decreasing the pH in the affected area, considering these values are inversely proportional.[33]

When analyzing the obtained results in the formalin test, an increase in time-spent licking and shaking behaviors was observed in phase 2 compared to phase 1 in the vehicle group, acetazolamide, sulfonamide, and the combinations of these with flavonoids. Supuran with others[34, 35] reported that flavonoids, as well as various phenolic compounds, acted by binding to the enzyme by anchoring to the hydroxyl ion, which was bound to the solvent molecule bound to zinc (Zn(II)). Based on these findings, it is expected that the effect of flavonoids on CA may be mediated by the inhibition of the activity of this enzyme on the hydration of CO_2 and the dehydration of H_2CO_3, which are reactions carried out by this enzyme, helping the pH of the medium to stabilize.[34, 35]

After the acetazolamide and sulfonamide inhibitors were given alone and in combination with the flavonoids (D/S, D/A, H/S, and H/A), no anti-inflammatory response was observed since the time-spent licking, the count of the number of shakings, and the

percentage of inflammation remained elevated without a special difference concerning the vehicle group. In fact, A and S reversed the activity of flavonoids when they were administered in combination. Like flavonoids, A and S are CA inhibitors that bind differently to the enzyme. Both lack isoform specificity, which can lead to side effects such as increased urination, dry mouth, nausea, and vomiting in the case of acetazolamide and diarrhea, abdominal pain, and headache in the case of sulfonamide.[36]

Supuran[34] evaluated the inhibition of CA with different natural products, reporting that inhibitors such as A and S acted by coordinating the inhibitor with the Zn(II) ion through the replacement of water/hydroxide ion linked to zinc, resulting in a tetrahedral geometry of Zn(II), thus interrupting the hydration cycle of carbon dioxide, which indicates that A and S are non-competitive inhibitors since the enzyme cannot catalyze its reaction, in addition to reducing the number of functional enzyme molecules that can perform the reaction. At this point, and considering that both A and S bind with the Zn(II) ion, change the conformation of the molecule, and replace the hydroxyl ion, we suppose that when flavonoids are administered, they bind to the hydroxyl ion and are displaced by the inhibitors A and S, which explains why no effect was observed. However, further studies are needed to evaluate the factors involved in the effect of A and S on D and H.

Moreover, it is known that A and S inhibitors have greater affinity and inhibitory activity on CAII and CAVII isoforms, which are considered therapeutic targets for pain.[37] This helps us to explain why a lack of anti-inflammatory activity was observed since CAII and CAVII are mainly located in the thalamus and cortex, leaving them very far from the hind limb, which was the site of injury. In contrast, the isoforms CAIV, CAIX, and CAXII are overexpressed in this site since they are present in the membrane of basal cells, i.e.,

in cells located in the lowest part (or base) of the epidermis, which is the outermost layer of the skin and with which the affinity of the inhibitors is lower.[38]

It has been reported that both A and S showed efficacy against different types of pain in doses ranging from 100 to 200 mg/kg in neuropathic and visceral pain models.[14, 39] However, concerning their anti-inflammatory effect, there are few studies, some of them related to their efficacy against chronic obstructive pulmonary disease, lung inflammation, and inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.[40] The reason may be because CAII is also located in the esophagus, colon, and stomach. Since A and S are more related to this isoenzyme, they contribute to alleviating diseases related to these organs.[38]

Although there is evidence that these inhibitors could produce an anti-inflammatory effect, more studies are needed to determine their efficacy in different types of inflammation since, particularly in the formalin test, it is suggested that the evaluated dose was not enough to inhibit all CAs present in the body, including ones mainly related to inflammation, and thus generate a high response in terms of the time-spent licking and the number of shakings. There are several *in vitro* and *in vivo* studies that highlight the action of A and S inhibitors in reducing symptoms related to neuropathic pain, presenting them as favorable drugs for this type of condition.[14] This, in the first place, makes it clear that both A and S can cross the blood-brain barrier and having greater affinity with the isoforms CAII and CAVII, which are in greater quantity in the thalamus, hippocampus, and cortex, as mentioned before.

Related to that, we could not find reports on their effects on inflammatory mechanisms. In this study, the injury was caused by a chemical stimulus (1 % formalin)

to cause inflammation of a limb, as opposed to neuropathic pain, in which partial damage is caused to peripheral or spinal nerves such as spinal nerve ligation and partial sciatic nerve ligation. Regarding the combinations of A and S with flavonoids, a significant difference in the time-spent licking compared to the vehicle group was observed. Even so, this effect was not sufficient to match the response of the flavonoids individually administered, as there was a significant difference between the diosmin and hesperidin groups with the combinations. Concerning the number of shakings, the combinations with A and S did not show a decrease or a significant difference with the vehicle group.

The above suggests the importance of the mechanism that the flavonoids diosmin and hesperidin have on CA to cause an anti-inflammatory effect, since, as mentioned before, this is not the only mechanism by which these substances cause a response. Therefore, as we obtained a similar response to the vehicle with these combinations, we can suggest that CA inhibition is one of the main mechanisms by which the flavonoids produce their effect. Moreover, these results suggest that both A and S inhibited CA isoforms by changing the enzyme structure, preventing flavonoids from causing their anti-inflammatory effect; nevertheless, more studies are needed to evaluate.

Concerning the ethanol-induced gastric injury in rats, the flavonoids diosmin and hesperidin produced a marked protective effect against gastric ulceration, obtaining a high percentage of gastroprotection compared to the meloxicam and vehicle groups. The drug meloxicam produced an area of ulceration notably greater than the flavonoids visualized by the hemorrhagic bands. It has been shown that several flavonoids produce effective anti-ulcerogenic activity that is comparable to that of clinically used anti-ulcer drugs.[41] Suárez et al.[42] analyzed hesperidin in different experimental models of gastric

ulcer, finding that this flavonoid showed an outstanding ability to reduce the ulceration rate. Similarly, Elshazly et al.[43] proposed that the gastroprotective effect of flavonoids was associated with a decrease in free and total gastric acidity and an increase in gastric pH, which could be related to CA inhibition. However, more evidence is needed to support this information, and more studies should be carried out.

In conclusion, diosmin and hesperidin are promising alternatives for treating pain and inflammation, potentially through the inhibition of carbonic anhydrase. The explanation of CA inhibition due to diosmin and hesperidin mostly is unexplored, and our current findings have created new questions on the effects and molecular mechanisms of CA. Therefore, this is a starting point for further research towards more specific assays on the mechanisms of action involved in these new therapeutic applications.

Data availability

All relevant data are within the manuscript and its supporting information files.

Acknowledgments

The authors thank and acknowledge Rocío Margarita Santos-Osnaya, MVZ Lucía Macías-Rosales, Francisco Sánchez-Bartéz, MD, Marisol Rivera-Huerta, MD, MVZ Mabel Cecilia Tinoco-Méndez, MVZ Diana Patricia Galván-Vela and MVZ Stephanie Uribe-López for their experience and technical assistance.

Funding statement

This research was funded by Universidad Nacional Autónoma de México (www.unam.mx), grant number PAPIIT IN217921, and PAIP 5000-9179, awarded to AI Carballo-Villalobos. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare regarding this publication.

Author contributions

Conceptualization: AI Carballo-Villalobos.

Data curation: AI Carballo-Villalobos.

Formal analysis: A Zapata-Arenas, R Bustamante-García.

Funding acquisition: AI Carballo-Villalobos.

Investigation: AX Muñiz-Alquicira.

Methodology: AX Muñiz-Alquicira.

Project administration: AI Carballo-Villalobos.

Resources: AE Gómez-Martínez, AI Carballo-Villalobos.

Supervision: A Zapata-Arenas and R Bustamante-García, AE Gómez-Martínez.

Validation: A Zapata-Arenas and R Bustamante-García.

Visualization: AE Gómez-Martínez.

Writing-original draft: AX Muñiz-Alquicira.

Writing-review and editing: AI Carballo-Villalobos.

References

1. Damon M, Flandre O, Michel F, Perdrix L, Labrid C, Crastes de Paulet A. Effect of chronic treatment with a purified flavonoid fraction on inflammatory granuloma in the rat. Study of prostaglandin E2 and F2 alpha and thromboxane B2 release and histological changes. Arzneimittelforschung. 1987;37(10):1149–1153.
2. Rao YK, Fang S-H, Tzeng Y-M. Antiinflammatory activities of flavonoids and a triterpene caffeate isolated from *Bauhinia variegata*. Phytotherapy Research. 2008;22(7):957-962. doi: 10.1002/ptr.2448.
3. Mittal M, Siddiqui MR, Tran K, Reddy, S. P., Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxidants & Redox Signaling. 2014;20(7):1126–1167. doi: 10.1089/ars.2012.5149.
4. Ji G, Li Z, Neugebauer V. Reactive oxygen species mediate visceral pain-related amygdala plasticity and behaviors. Pain. 2015;156(5):825–836. doi: 10.1097/j.pain.0000000000000120.
5. Wang J, Cochran V, Abdi S, Chung JM, Chung K, Kim HK. Phenyl N-t-butylnitrone, a reactive oxygen species scavenger, reduces zymosan-induced visceral pain in rats. Neuroscience Letters. 2008;439(2):216–219. doi: 10.1016/j.neulet.2008.05.018.
6. Ferraz CR, Carvalho TT, Manchope MF, Artero NA, Rasquel-Oliveira FS, Fattori V, et al. Therapeutic potential of flavonoids in pain and inflammation: mechanisms of action, pre-clinical and clinical data, and pharmaceutical development. Molecules. 2020;25(3):762. doi: 10.3390/molecules25030762.

7. Zheng Y, Zhang R, Shi W, Li L, Liu H, Chen Z, et al. Metabolism and pharmacological activities of the natural health-benefiting compound diosmin. Food & Function. 2020;11(10):8472–8492. doi: 10.1039/d0fo01598a.

8. Carballo-Villalobos AI, González-Trujano ME, Pellicer F, Alvarado-Vásquez N, López-Muñoz FJ. Central and peripheral anti-hyperalgesic effects of diosmin in a neuropathic pain model in rats. Biomedicine & Pharmacotherapy. 2018;97:310–320. doi: 10.1016/j.biopha.2017.10.077.

9. Bertozzi MM, Rossaneis AC, Fattori V, Longhi-Balbinot DT, Freitas A, Cunha FQ, et al. Diosmin reduces chronic constriction injury-induced neuropathic pain in mice. Chemico-Biological Interactions. 2017;273:180–189. doi: 10.1016/j.cbi.2017.06.014.

10. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nature Reviews Drug Discovery. 2008;7(2):168–181. doi: 10.1038/nrd2467.

11. Mishra CB, Kumari S, Angeli A, Bua S, Tiwari M, Supuran CT. Discovery of benzenesulfonamide derivatives as carbonic anhydrase inhibitors with effective anticonvulsant action: design, synthesis, and pharmacological evaluation. Journal of Medicinal Chemistry. 2018;61(7):3151–3165. doi: 10.1021/acs.jmedchem.8b00208.

12. Taslimi P, Caglayan C, Gulcin İ. The impact of some natural phenolic compounds on carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase, and α-glycosidase enzymes: an antidiabetic, anticholinergic, and antiepileptic study.

Journal of Biochemical and Molecular Toxicology. 2017;31(12):e21995. doi: 10.1002/jbt.21995.

13. Carballo-Villalobos AI, González-Trujano ME, Pellicer F, López-Muñoz FJ. Antihyperalgesic effect of hesperidin improves with diosmin in experimental neuropathic pain. BioMed Research International. 2016;2016(1):8263463. doi: 10.1155/2016/8263463.
14. Asiedu M, Ossipov MH, Kaila K, Price TJ. Acetazolamide and midazolam act synergistically to inhibit neuropathic pain. Pain. 2010;148(2):302–308. doi: 10.1016/j.pain.2009.11.015.
15. Carta F, Supuran CT, Scozzafava A. Sulfonamides and their isosters as carbonic anhydrase inhibitors. Future Medicinal Chemistry. 2014;6(10):1149–1165. doi: 10.4155/fmc.14.68.
16. Sarkhel S. Evaluation of the anti-inflammatory activities of *Quillaja saponaria* Mol. saponin extract in mice. Toxicology Reports. 2016;3:1–3. doi: 10.1016/j.toxrep.2015.11.006.
17. Bharani S, Naik A, Parija S, Panda S. Meloxicam induced toxicopathology studies in Wistar rats. Indian Journal of Animal Research. 2019;54(3):363–366. doi: 10.18805/ijar.B-3766.
18. Robert A, Nezamis JE, Lancaster C, Hanchar AJ. Cytoprotection by prostaglandins in rats: prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. Gastroenterology. 1979;77(3):433–443. doi: 10.1016/0016-5085(79)90002-7.

19. Wheeler-Aceto H, Cowan A. Standardization of the rat paw formalin test for the evaluation of analgesics. Psychopharmacology. 1991;104(1):35–44. doi: 10.1007/bf02244551.

20. Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain. 1992;51(1):5–17. doi: 10.1016/03043959(92)90003-t.

21. McCall WD, Tanner KD, Levine JD. Formalin induces biphasic activity in C-fibers in the rat. Neuroscience Letters. 1996;208(1):45-48. doi: 10.1016/0304-3940(96)12552-0.

22. Khalil NY, Aldosari KF. Meloxicam. In: HG Brittain, editor. Chapter Six - Meloxicam, Profiles of Drug Substances, Excipients and Related Methodology, 45. Riyadh, Saudi Arabia: Academic Press; 2020. pp. 159–197. ISSN 1871-5125, ISBN 9780128203231.

23. Euchenhofer C, Maihöfner C, Brune K, Tegeder I, Geisslinger G. Differential effect of selective cyclooxygenase-2 (COX-2) inhibitor NS 398 and diclofenac on formalin-induced nociception in the rat. Neuroscience Letters. 1998;248(1):25–28. doi: 10.1016/S0304-3940(98)00325-5.

24. Serafini M, Peluso I, Raguzzini A. Flavonoids as anti-inflammatory agents. Proceedings of the Nutrition Society. 2010;69(3):273–278. doi: 10.1017/s002966511000162x.

25. Bouskela E, Cyrino FZGA, Lerond L. Effects of oral administration of different doses of purified micronized flavonoid fraction on microvascular reactivity after

ischaemia/reperfusion in the hamster cheek pouch. British Journal of Pharmacology. 1997;122(8):1611–1616. doi: 10.1038/sj.bjp.0701554.

26. Vabeiryureilai M, Lalrinzuali K, Jagetia GC. Chemopreventive effect of hesperidin, a citrus bioflavonoid in two stage skin carcinogenesis in Swiss albino mice. Heliyon. 2019;5(10):e02521. doi: 10.1016/j.heliyon.2019.e02521.

27. Boonpawa R, Spenkelink A, Punt A, Rietjens IMCM. Physiologically based kinetic modeling of hesperidin metabolism and its use to predict *in vivo* effective doses in humans. Molecular Nutrition & Food Research. 2017;61(8):1600894. doi: 10.1002/mnfr.201600894.

28. García Meijide JA, Gómez-Reino Carnota J. Fisiopatología de la ciclooxigenasa-1 y ciclooxigenasa-2. Revista Española de Reumatología. 2000;27(1):33–35.

29. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nature Reviews Drug Discovery. 2008;7(2):168–181. doi: 10.1038/nrd2467.

30. Margheri F, Ceruso M, Carta F, Laurenzana A, Maggi L, Lazzeri S, et al. Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium. Journal of Enzyme Inhibition and Medicinal Chemistry. 2016;31(sup4):60–63. doi: 10.1080/14756366.2016.1217857.

31. Supuran CT. Carbonic anhydrase inhibition and the management of neuropathic pain. Expert Review of Neurotherapeutics. 2016;16(8):961–968. doi: 10.1080/14737175.2016.1193009.

32. Steen KH, Steen AE, Reeh PW. A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, *in vitro*. Journal of Neuroscience. 1995;15(5):3982–3989. doi: 10.1523/jneurosci.15-05-03982.1995.

33. Supuran CT. Carbonic anhydrase versatility: from pH regulation to CO_2 sensing and metabolism. Frontiers in Molecular Biosciences. 2023;10:1326633. doi: 10.3389/fmolb.2023.1326633.

34. Supuran CT. Carbonic anhydrase inhibition with natural products: novel chemotypes and inhibition mechanisms. Molecular Diversity. 2011;15(2):305–316. doi: 10.1007/s11030-010-9271-4.

35. Karioti A, Carta F, Supuran CT. Phenols and polyphenols as carbonic anhydrase inhibitors. Molecules. 2016;21(12):1649. doi: 10.3390/molecules21121649.

36. Aggul AG, Uzun N, Kuzu M, Taslimi P, Gulcin I. Some phenolic natural compounds as carbonic anhydrase inhibitors: an *in vitro* and *in silico* study. Archiv der Pharmazie. 2022;355(6):2100476. doi: 10.1002/ardp.202100476.

37. Kılıcaslan S, Arslan M, Ruya Z, Bilen Ç, Ergün A, Gençer N, et al. Synthesis and evaluation of sulfonamide-bearing thiazole as carbonic anhydrase isoforms hCA I and hCA II. Journal of Enzyme Inhibition and Medicinal Chemistry. 2016;31(6):1300–1305. doi: 10.3109/14756366.2015.1128426.

38. Espinosa L Sierra MP. Anhidrasa carbónica, nuevas perspectivas. Neumología y Cirugía de Tórax. 2010;69(4):200–209.

39. Asiedu MN, Mejia GL, Hübner CA, Kaila K, Price TJ. Inhibition of carbonic anhydrase augments $GABA_A$ receptor-mediated analgesia via a spinal mechanism

of action. The Journal of Pain. 2014;15(4):395–406. doi: 10.1016/j.jpain.2014.01.001.

40. Kelly TE, Hackett PH. Acetazolamide and sulfonamide allergy: a not so simple story. High Altitude Medicine & Biology. 2010;11(4):319–323. doi: 10.1089/ham.2010.1051.

41. Mehreen T, Aamir S, Shah NA, Shahid M, Javaid I, Roghani M, et al. Effect of the flavonoid 6-aminoflavone in aspirin induced gastric ulcer in sprague-dawely rats: a histomorphological study. Journal of Ayub Medical College Abbottabad. 2022;34(4)(Suppl 1):940–943. doi: 10.55519/jamc-04-s4-10369.

42. Suárez J, Herrera MD, Marhuenda E. Hesperidin and neohesperidin dihydrochalcone on different experimental models of induced gastric ulcer. Phytotherapy Research. 1996;10(7):616–618. doi: 10.1002/(SICI)1099-1573(199611)10:7<616::AID-PTR897>3.0.CO;2-N.

43. Elshazly SM, Abd El Motteleb DM, Ibrahim I. Hesperidin protects against stress induced gastric ulcer through regulation of peroxisome proliferator activator receptor gamma in diabetic rats. Chemico-Biological Interactions. 2018;291:153–161. doi: 10.1016/j.cbi.2018.06.027.