

RESEARCH ARTICLE

Functional mechanism of tracheal relaxation, antiasthmatic, and toxicological studies of 6-hydroxyflavone

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Abstract

Previously, we described tracheal rat rings relaxation by several flavonoids, being 6-hydroxyflavone (6-HOF) the most active derivative of the series. Thus, its mechanism of action was determined in an ex vivo tracheal rat ring bioassay. The anti-asthmatic effect was assayed in in vivo OVALbumin (OVA)-sensitized guinea pigs. Finally, the toxicological profile of 6-HOF was studied based on Organization of Economic Cooperation and Development guidelines with modifications. 6-HOF-induced relaxation appears to be related with receptor-operated calcium channel and voltage-operated calcium channel blockade as the main mechanism of action, and also through the production of relaxant second messengers NO and cGMP. Molecular docking supports that 6-HOF acts as calcium channel blocker and by activation of nitric oxide synthase. In addition, the in vivo anti-asthmatic experiments demonstrate the dose-dependent significant anti-allergic effect of 6-HOF induced by OVA, with best activity at 50 /kg. Finally, toxicological studies determined a LD₅₀ > 2,000 mg/kg and, after 28 day of treatment with 6-HOF (50 mg/kg) by intragastric route, mice did not exhibit evidence of any significant toxicity. In conclusion, experiments showed that 6-HOF exerts significant relaxant activity through calcium channel blockade, and possibly, by NO/cGMP-system stimulation on rat trachea, which interferes with the contraction mechanism of smooth muscle cells in the airways. In addition, the flavonoid shows potential anti-asthmatic properties in an anti-allergic pathway. Furthermore, because the pharmacological and safety evidence, we propose this flavonoid as lead for the development of a novel therapeutic agent for the treatment of asthma and related respiratory diseases.

KEYWORDS

6-hydroxyflavone, allergy, asthma, calcium channel blockade, toxicology, tracheal relaxation

Abbreviations: 2-AP, 2-aminopyridine; 6-HOF, 6-hydroxyflavone; AHR, airway hyperresponsiveness; ALT, alanine aminotransferase; ANOVA, analysis of variance; ASM, airway smooth muscle; AST, aspartate aminotransferase; CaCl₂, calcium chloride; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CRC, concentration–response curve; DMSO, dimethyl sulfoxide; eNOS, endothelial nitric oxide synthase; EPO, eosinophil peroxidase; H&E, hematoxylin and eosin; HDL, high-density lipoprotein cholesterol; IgE, immunoglobulin E; IL, interleukin; KCl, potassium chloride; KH₂PO₄, potassium phosphate monobasic; L-NAME, N(ω)-nitro-L-arginine methyl ester; MgSO₄, magnesium sulfate; NaCl, sodium chloride; NaHCO₃, sodium bicarbonate; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one; OECD, Organization for Economic Cooperation and Development; OVA, ovalbumin; PKA, protein kinase A; ROC, receptor-operated calcium channels; ROW, relative organ weight; SEM, standard error of the mean; sGC, soluble guanylyl cyclase; SOCE, store-operated calcium entry; VOC, voltage-operated calcium channels.

[†]Taken in part from the PhD Dissertation of A. Flores-Flores.

1 | INTRODUCTION

Allergic asthma is a chronic airway inflammatory disease that manifests itself as recurrent reversible bronchoconstriction and airway hyperresponsiveness (AHR) (GINA, 2017). It is associated with changes in the levels of eosinophils, mast cells, lymphocytes, cytokines, and other inflammatory cell products. Currently, asthma therapy lacks satisfactory success because of the adverse effects of bronchodilators and anti-inflammatory drugs, and patients resort to alternative medicine to treat their asthma (GINA, 2017). In this regard, research for novel active plant compounds has increased, considering that natural products offer a structural diversity with different pharmacological effects and specific mechanisms of action, and in several cases, that are accompanied by multitarget interactions. A clear example are flavonoids, which are small molecules, and their classification depends on patterns of substitution in the heterocyclic ring, such as chalcones, flavones, flavonols, flavanols, flavans, flavanones, anthocyanidins, and isoflavonoids, and their different structures lead to changes in bioactivity and metabolism (Havsteen, 2002; Wen et al., 2017). Recent reports show that more than 9,000 flavonoids possess important effects, including antiviral/bacterial, antidiabetic, anticancer, antiaging (Wang, Li, & Bi, 2018), anti-allergic, anti-hepatotoxic, and antispasmodic activities, and protection against cardiovascular events (Harborne & Williams, 2000; Havsteen, 2002; Torres-Piedra et al., 2011; Wang et al., 2018; Wen et al., 2017). Also, they demonstrate vasorelaxant, antioxidant, antiatherogenic, and bronchodilatory activities, among others (Harborne & Williams, 2000; Tanaka & Takahashi, 2013; Torres-Piedra et al., 2011). In addition, flavonoids have been suggested as having anti-inflammatory properties, which avoid damage induced by neutrophils as the central cell in the acute inflammatory process (Zeinali, Rezaee, & Hosseinzadeh, 2017). Moreover, the anti-allergic activity of fisetin was shown to inhibit the degranulation of immunoglobulin E (IgE)/Ag-stimulated RBL-2H3 cells in a dose-dependent manner (Jo & Park, 2017). In addition, in an ovalbumin (OVA)-sensitized mouse model, apigenin, when administered intraperitoneally (i.p.) at 5 or 10 mg/kg before the last OVA challenge, resulted in a significant inhibition of asthmatic reactions, such as serum IgE elevation, eosinophil accumulation, IL-4, IL-5, and eosinophil peroxidase activity in BALF, as well as AHR. Thus, the aim of this study was to expand previous findings, emphasizing whether **6-HOF** causes tracheal relaxation and to characterize the putative pharmacological mechanisms responsible for such an effect. In addition, we sought to establish the *in vivo* antiasthmatic effect in an OVA-induced allergic model and the toxicological profile of **6-HOF** in acute and sub-chronic mouse models.

2 | MATERIALS AND METHODS

2.1 | Chemicals and drugs

Carbamoylcholine chloride (carbachol), theophylline, glibenclamide, 2-aminopyridine, *N*-(ω)-nitro-L-arginine methyl ester (L-NAME), 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), indomethacin, Iso-proterenol, Nifedipine, CaCl₂, 6-hydroxyflavone (**6-HOF**), KCl, OVA,

aluminum hydroxide, dimethyl sulfoxide, and propylenglycol were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other reagents were of analytical-grade and from a local source. Stock solutions of **6-HOF** were formulated utilizing distilled water and were freshly prepared on the same day of experimentation.

2.2 | Animals

Male Wistar rats (weighing 250–300 g) and male ICR mice (weighing 25–30 g) were obtained from FESI Bioterium, Universidad Nacional Autónoma de México. Male, outbred guinea pigs (weighing 350–400 g) were purchased from Harlan-México (strain HsdPoc:DH). The animals were maintained under filtered, air-conditioned, 12-/12-hr light/dark cycles at 21 ± 1 °C and at 50–70% humidity and were fed sterilized pellets (Purina Chow or 2040 Harlan Teklad Guinea Pig Diet) and water *ad libitum*.

All of the animals' procedures were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, México) and were approved by the Scientific and Bioethics Committee of the Instituto Nacional de Enfermedades Respiratorias with assigned code B01-16.

2.3 | Isolated thoracic tracheal rat ring model

The Wistar rats were killed by cervical dislocation. The trachea was dissected and cleaned of connective tissue and mucus, and immediately sectioned into rings 4–5-mm in length (each containing 2–3 cartilaginous rings). Then, tissue segments were mounted on stainless steel hooks under an optimal tension of 2 g in 10-ml organ baths containing warmed (37 °C) and oxygenated (O₂/CO₂, 95:5) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0, and glucose, 11.1, pH 7.4). Changes in tension were recorded by Grass-FT03 force transducers (Astromed, West Warwick, RI) connected to an MP100 Analyzer (Biopac Instruments, Santa Barbara, CA) as described (Estrada-Soto et al., 2012). After equilibration, the rings were contracted by carbachol (1 μ M) and washed every 40 min for 2 hr. After contraction with carbachol, the test samples (**6-HOF**, vehicle, and positive control) were added at the bath in a volume of 100 μ L; then, cumulative concentration-response curves (CRC) were obtained for each ring. The relaxant effect of the test samples was determined by comparing the muscular tone of the contraction before and after addition of the test materials. Muscular tone was calculated from the tracings using Acknowledge software (Biopac®).

2.3.1 | Determination of the underlying functional mode of action of 6-HOF on tracheal rat rings

The following *ex vivo* experiments were carried out:

- For interaction with the cholinergic receptors, CRC were obtained with carbachol (0.006–540 μ M) after the tissues were incubated with 47 or 84.14 μ M of **6-HOF** during 15 min. The carbachol-contraction effect was determined by comparing the contraction induced by carbachol in the absence and presence of **6-HOF**.

- For interaction with the β -adrenergic receptor, tissues were preincubated during 15 min with Isoproterenol (10 μ M; β -adrenergic agonist), and the maximal relaxing effect of **6-HOF** was compared in the absence and presence of Isoproterenol.
- To establish a possible interaction of **6-HOF** with the L-type calcium channel, tracheal rings were washed with Krebs solution, then rinsed with a solution containing KCl (80 mM). Once a plateau was attained, CRC of **6-HOF**-induced relaxation were obtained by adding cumulative concentrations of the compound to the bath.
- To determine whether the inhibition of extracellular Ca^{2+} influx was involved in **6-HOF**-induced relaxation, the experiments were carried out in Ca^{2+} -free Krebs solution. Tracheal rings were washed with Ca^{2+} -free Krebs solution containing KCl (80 mM) (15 min), and cumulative CRC for CaCl_2 were obtained in the absence of **6-HOF** (control group) or after a 15-min incubation with **6-HOF** (26.3, 47, 84.14, and 149.88 μ M). Finally, the contractile effect induced by CaCl_2 was compared in the absence and presence of **6-HOF**.
- For disruption of the NO/cGMP pathway, the tissues were incubated with L-NAME (10 μ M, NOS inhibitor) or ODQ (10 μ M, sGC inhibitor) during 15 min, and immediately, **6-HOF** was added at different concentrations and cumulative CRC were obtained.

2.4 | Docking studies

Human endothelial nitric oxide synthase (eNOS) (1M9R, PDB code; Berman et al., 2000) and human L-type calcium channel, modeled by Lipkind and Fozzard (2003), were used as reference target. Docking standardization for each target was carried out utilizing previously bound or docked molecules, that is, the 3-bromo-7-nitroindazole structure bound to the crystallographic structure, 1M9R, was employed for NOS. Nifedipine was used for the human L-type calcium channel ligand. Nifedipine and **6-HOF** were constructed using MarvinSketch by ChemAxon (2018). 3-Bromo-7-nitroindazole coordinates were obtained from the crystallographic structure, 1M9R. A validated protocol was used to reproduce correctly the docking root-mean-square deviation (RMSD) between 3-bromo-7-nitroindazole and 1M9R crystal ligand. The RMSD obtained was 2.66 ± 0.49 Å by 100 independent docking experiments.

Docking experiments were directed to the known binding site of each target. The NOS grid-box center was located at (24.16, 12.55, 30.29) with a size of $14 \times 14 \times 24$ Å³; this box was sufficient to cover S, M, C2, and C1 sites, as reported by Ríos et al. (2012). The L-type calcium channel grid center was at (0, 0, 0) with a size of $22.50 \times 22.50 \times 22.50$ Å³. All grid boxes had 1 Å of grid spacing. All systems were prepared utilizing PyMol 2.1.0 by Schrödinger (2010) and the Autodock Vina plug-in for PyMol (Seeliger & de Groot, 2010). Autodock Vina (Trott & Olson, 2010) software was used to conduct all docking studies; 100 independent docking runs for each ligand were performed.

All images were performed using Visual Molecular Dynamics (Humphrey, Dalke, & Schulten, 1996), and protein–ligand interaction maps were created with Maestro (Schrödinger, 2018).

2.5 | In vivo experiments in the guinea pig asthma model induced by an OVA-sensitization procedure

On Day 1, the guinea pigs received a mixture of 60 μ g/ml of OVA with aluminum hydroxide (1 mg/ml) dispersed in saline solution via both i.p. (0.5 mg/ml) and subdermal (s.d.) (0.5 mg/ml) injections. Antigen sensitization was reinforced 8 days later with OVA aerosol (3 mg/ml) delivered during 5 min. The aerosols were produced by a US-1 Bennett Nebulizer (flow, 2 ml/min; Multistage Liquid Impinger; Burkard Manufacturing Co., Rickmansworth, Hertfordshire, UK), releasing mixed particles <4 μ m (44%), 4–10 μ m (38%), and > 10 μ m (18%) in size. On Day 15, guinea pigs that developed immediate airway obstruction (the asthma model) after antigen challenge during 1 min with OVA aerosol (1 mg/ml) were used for **6-HOF** and vehicle treatments. Additional control groups of non-sensitized guinea pigs were included.

2.6 | Barometric plethysmography

Inhalation challenges were carried out by using a barometric plethysmograph to record acute airway obstructive response to the antigen immediately after OVA delivery. We utilized whole-body single-chamber plethysmography for freely moving animals (Buxco Electronics, Inc., Troy, NY) to evaluate pulmonary function according to the principles previously described.

2.7 | Determination of bronchial obstruction

6-HOF was tested at different doses (30, 50, and 90 mg/kg), or propylenglycol (10%) was injected i.p. into animals 45 min before the OVA aerosol challenge. Guinea pigs were challenged for 1 min, and bronchial obstruction was recorded for one additional minute for each increasing dose of aerosolized OVA or saline aerosol. The highest value on the bronchial obstruction (iB) index obtained during each OVA challenge was expressed as a percentage of the baseline iB value exhibited after saline challenge, to determine the potential bronchodilatory effect of **6-HOF**.

2.8 | Toxicological studies

2.8.1 | Acute oral toxicity test

The acute toxicity study for **6-HOF** was carried out to evaluate any possible toxicity. The experiment was conducted in accordance with the recommendation of Organization for Economic Cooperation and Development (OECD) Guideline 425 (OECD, 2008). For this purpose, three male ICR mice (weighing 25–30 g), orally administered with gradual doses (5, 50, 300, and 2,000 mg/kg body weight), were used. After treatment, the animals were carefully observed for signs of toxicity, mortality, and general behavior for 4 hr. Animals were also monitored for 14 days after treatment.

2.8.2 | Sub-chronic toxicity study

The sub-chronic experiment was carried out using repeated doses of **6-HOF** (Hernández-Vázquez et al., 2017; Parasuraman, 2011). Two independent groups of six male ICR mice (weighing 25–30 g each) were administered daily with **6-HOF** (50 mg/kg) oral dose by gavage

for 28 days, and the control group received saline solution. All animals survived until the end of the observation period. Visible and transitory signs of toxicity were carefully observed during the first 4 hr following each administration, as well as at 8 and 12 hr afterward. Changes in body weight were monitored daily during the entire study.

2.8.3 | Relative organ weight

The weight of the organs of all of the mice was recorded at the end of the experiment. The kidneys, liver, lungs, and heart of each mouse in the experimental and control groups were collected and weighed. Relative organ weight (ROW) was calculated as follows: $ROW = (\text{organ weight/body weight}) \times 100$.

2.8.4 | Determination of biochemical parameters

Blood was collected by cardiac puncture. The blood sample was maintained at room temperature for 30 min for it to clot. Afterward, the test tube containing the clotted blood sample was centrifuged at 10,000 rpm for 10 min to obtain serum. The serum was used for the biochemical tests. The values of blood glucose, cholesterol, triglycerides, HDL, ALT, and AST were determined following standard laboratory procedures.

2.8.5 | Histopathological examination

A complete necropsy was performed on each animal, in which a careful macroscopic observation inspected for the presence or absence of anatomic abnormalities was carried out, comparing treated and control groups. Tissue samples that were collected from the lungs, liver, kidneys, and heart of each animal were fixed immediately by immersion in 10% buffered formalin, then routinely processed and embedded in paraffin. The paraffin-embedded tissues were sectioned at a width of 4–5 μm , mounted on glass slides, and stained with hematoxylin and eosin (H&E). Histological slides were examined with a conventional light microscope (Zeiss 40 $\times$).

2.9 | Statistical analysis

Pharmacological data was analyzed by two-way analysis of variance and post hoc Tukey and Dunnett tests. The difference was considered significant with a p value of $<.05$. All values were expressed as the mean $\pm$ the standard error of the mean. CRC were plotted, and the obtained experimental data were adjusted by a nonlinear curve-fitting program (ORIGIN 8.0).

3 | RESULTS AND DISCUSSION

Natural products are an important source for discovery and development of new drugs with novel therapeutic targets for more specific treatments and less adverse effects. Within this context, flavonoids are privileged scaffolds that possess several activities and mechanisms of action (Goszcz, Duthie, Stewart, Leslie, & Megson, 2017; Harborne & Williams, 2000; Wang et al., 2018). In previous reports, the potential effect of flavonoids as antiasthmatic drugs, especially those possessing powerful antioxidant, anti-allergic, anti-inflammatory, immune-modulating, and airway smooth muscle relaxant effects, have

been described (Flores-Flores et al., 2018; Goszcz et al., 2017; Tanaka & Takahashi, 2013; Wang et al., 2018). The majority of these induce their antiasthmatic effect via the inhibition of the release of chemical mediators, the synthesis of Th2 type cytokines such as interleukin (IL)-4 and IL-13, and CD40 ligand expression by high-affinity IgE receptor-expressing cells such as mast cells and basophils (Tanaka & Takahashi, 2013). They also inhibit IL-4-induced signal transduction and affect the differentiation of naïve CD4 $^{+}$ T cells into effector T cells through their inhibitory effect on the activation of the aryl hydrocarbon receptor (Tanaka & Takahashi, 2013). Various studies of flavonoids in asthmatic animal models have demonstrated the beneficial effects of luteolin, apigenin, and fisetin, among others (Tanaka & Takahashi, 2013). They significantly suppressed OVA-induced airway bronchoconstriction and AHR in Balb-c mice. It is well known that several antiasthmatic drugs have exhibited an adverse effect and/or they cannot efficiently control the disease and its related symptoms. Thus, for treatment, the combinatorial therapeutic strategy with bronchodilators and anti-inflammatory drugs is required. Within this context, our research group is dedicated to discovering and developing new dual drugs that are capable of treating asthma from the bronchodilatory and anti-inflammatory points-of-view. Thus, in the current work, we describe how **6-HOF** induced its relaxant effect on tracheal rat rings (underlying the functional mode of action), as well as its significant suppression of OVA-induced airway bronchoconstriction in a guinea pig model.

3.1 | Tracheal relaxation and its underlying mechanism of action: functional and in silico experiments

In a previous work (Flores-Flores et al., 2018), the authors described that **6-HOF** induced significant relaxation on isolated tracheal rat rings precontracted with carbachol. In fact, **6-HOF** was the most potent compound of the entire series studied; thus, in the current investigation, **6-HOF** induced significant concentration-dependent relaxation that was more potent than that of the positive control (theophylline) (Figure 1a). Moreover, preincubation with 47 and 84.14 μM of **6-HOF** diminished, and in nonparallel and concentration-dependent manners, the contractions induced by carbachol and significantly depressed the maximal response (Figure 1b). Recent results suggest that the flavonoid-relaxant effect could be produced by a noncompetitive antagonism by means of cholinergic-receptor allosteric-site interaction and/or by a functional antagonism associated with a β -adrenoceptor agonism, second-messenger accumulation, and/or calcium channel blockade (Alemán-Pantitlán et al., 2016; Sánchez-Recillas et al., 2014). Therefore, we decided to elucidate the functional mode of action of **6-HOF** as a functional antagonist on those targets.

Asthma is characterized by excessive airway smooth muscle (ASM) contraction. ASM contraction is dependent on the phosphorylation of the regulatory myosin light-chain mediated by myosin light-chain kinase, which is in turn activated by the binding of calmodulin and calcium (Chen & Sanderson, 2017; Pfitzer, 2001). Hence, an increase of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) induces the contraction of ASM; in this regard, the flavonoid relaxed the contraction induced by KCl 80 mM (Figure 2a), similar to Nifedipine (an L-

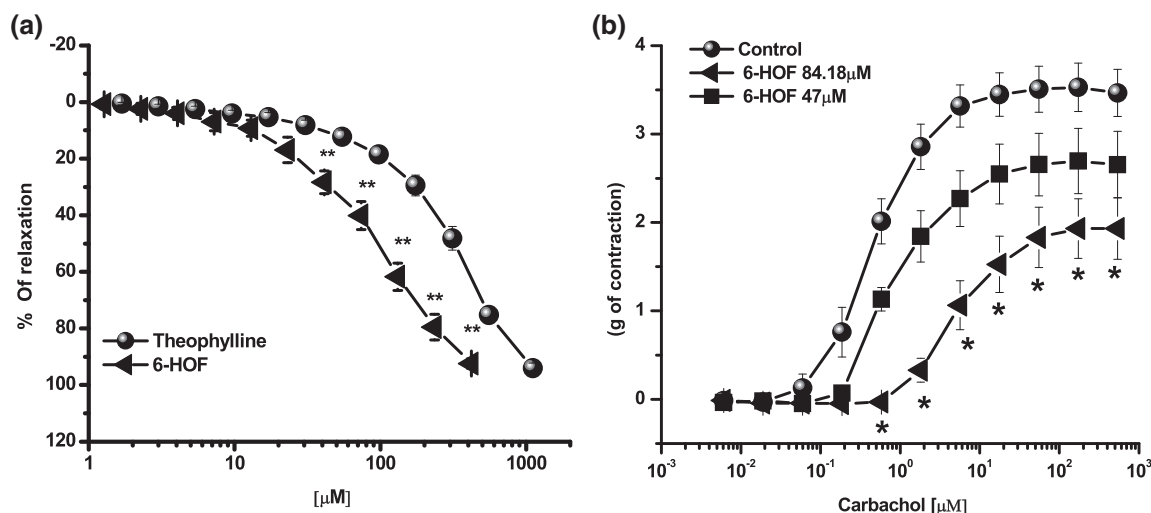


FIGURE 1 (a) Relaxing concentration–response curves (CRC) of 6-hydroxyflavone (**6-HOF**) on tracheal rat rings contracted with carbachol (1 μ M). (b) CRC of carbachol in the absence and presence of increasing concentrations of **6-HOF**. Data are expressed as the mean $\pm$ the standard error of the mean of six experiments (** $p < .01$; * $p < .05$)

type calcium channel blocker), employed as positive control; while the CaCl_2 -induced contraction was significantly reduced by the **6-HOF** (Figure 2b), suggesting that the flavonoid acts as a calcium channel blocker (Godfraind, 2017). The contraction of smooth muscle induced by high K^+ is dependent on the entry of Ca^{2+} into the cells through voltage-operated calcium channel (VOC) cell-membrane depolarization, while inhibition of high K^+ -induced contraction is because of the result of blocked Ca^{2+} entry through these VOC (Ghayur, Gilani, Khan, Amor, & Choudhary, 2006; Godfraind, 2017; Yagi, Kuwahara, & Tsunobone, 2002). Morel and Godfraind described that, in the presence of dihydropyridines, KCl-dependent contraction is lowered as a function of the concentration of the blocker and duration of the depolarization, implying that membrane potential is involved in dihydropyridine action, such as that revealed by Nifedipine (Figure 2a) (Morel & Godfraind, 1987). Thus, a variety of Ca^{2+} entry pathways are proposed to contribute to Ca^{2+} homeostasis in ASM contraction, including VOC, receptor-operated Ca^{2+} channels (ROC) and reverse-mode $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Chen & Sanderson, 2017). However, the most important

Ca^{2+} influx pathway coupled with Ca^{2+} loss from sarcoplasmic reticulum is probably that of store-operated calcium entry (SOCE). SOCE has been recently identified as a Ca^{2+} influx pathway in ASM contraction (Chen & Sanderson, 2017). Because **6-HOF** was capable of relaxing the contraction induced by KCl 80 mM, and to avoid CaCl_2 - and carbachol concentration-dependent contractions, the data suggested that flavonoid might obstruct both ROC and VOC (Godfraind, 2017). However, it is necessary to conduct more experiments to discard or include the participation of SOCE.

However, the role of second messengers NO, cGMP, and cyclic adenosine monophosphate (cAMP) production and/or accumulation and potential β -adrenoceptor agonism by **6-HOF** as relaxant mechanism was investigated. In the presence of L-NAME (an unspecific NOS inhibitor) and ODQ (a specific soluble guanylyl-cyclase inhibitor), **6-HOF** relaxant curves were significantly displaced to the right and the efficacies were drastically diminished (Figure 3a), indicating that a second mechanism of action is involved in **6-HOF** actions, such as possible increases of NO/cGMP (Chen & Ko, 2017). Recent

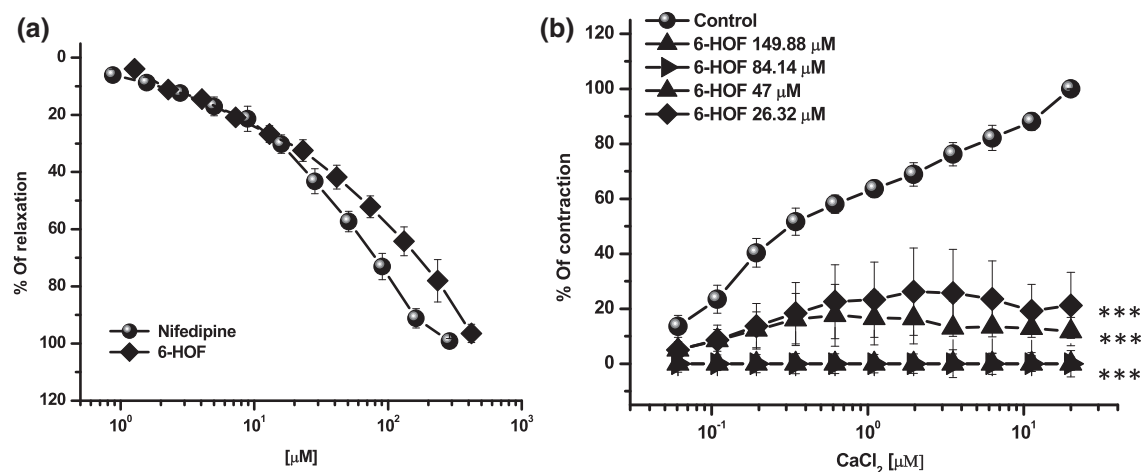
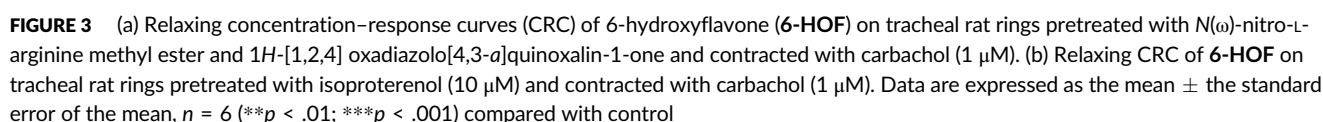
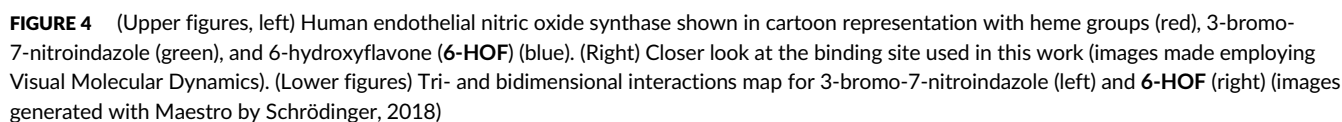


FIGURE 2 (a) Relaxing concentration–response curves of 6-hydroxyflavone (**6-HOF**) on tracheal rat rings contracted with KCl (80 mM). (b) Inhibitory effect of **6-HOF** on the contraction curves induced by the cumulative concentration of extracellular CaCl_2 in Ca^{2+} -free solution. Results are presented as the mean $\pm$ the standard error of the mean, $n = 6$. *** $p < .001$ each point compared with control



Kramp, Harris, & van den Akker, 2016). However, it is well known that the activation of the β -adrenoceptor coupled to Gs-protein, by its β -agonists, involves the production of cAMP through the activation of adenylate cyclase, which induces bronchodilation and anti-inflammatory actions on airways through the activation of PKA (Barnes, 1995; Hamad et al., 2003). However, the effect of **6-HOF** in the presence of isoproterenol was not modified (Figure 3b), which allowed us to discard β -adrenoceptor agonism and a possible involvement of cAMP production in the relaxant effect of the flavonoid.



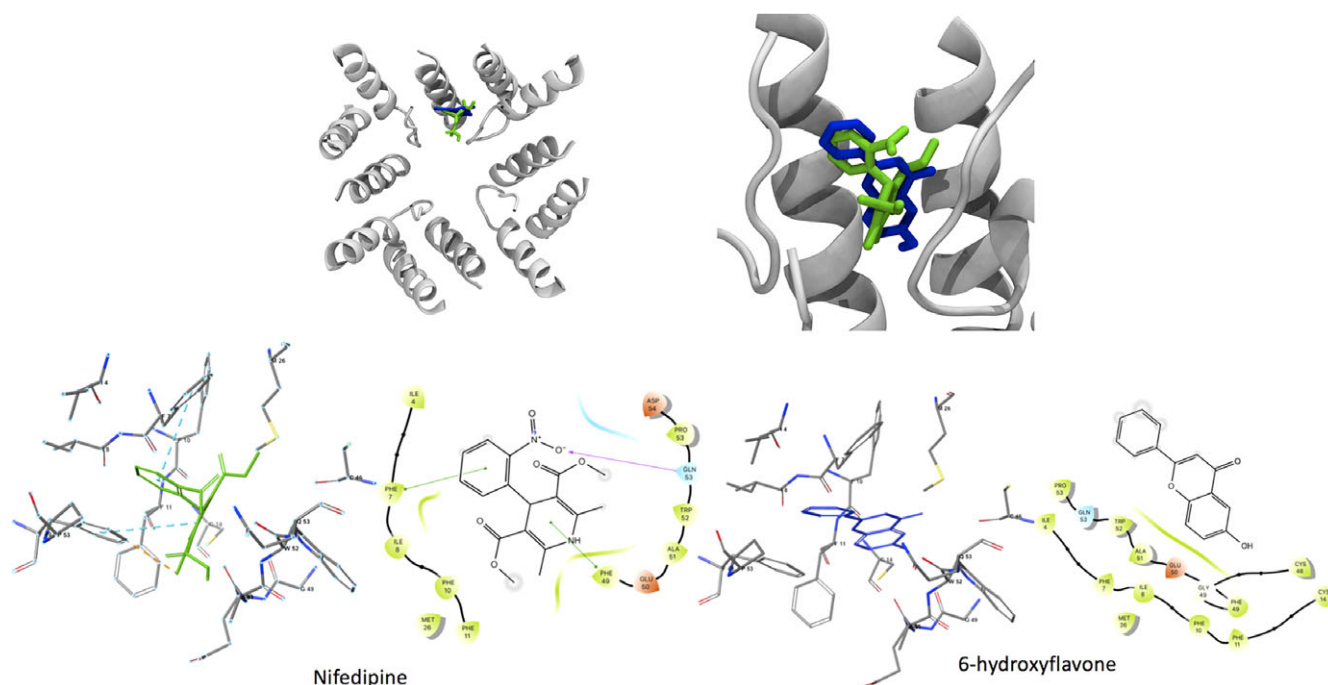


FIGURE 5 (Upper figures, left) L-type human calcium channel model shown in cartoon representation with Nifedipine (green) and 6-hydroxyflavone (**6-HOF**) (blue). (Right) Closer look at the binding site used in this work (images made employing Visual Molecular Dynamics). (Lower figures) Tri- and bidimensional interactions maps for Nifedipine (left) and **6-HOF** (right) (images generated with Maestro by Schrödinger, 2018)

Because the relaxant effect of **6-HOF** is related with a calcium channel blockade and was greatly reduced by L-NAME (an established NOS inhibitor), we decided to investigate the *in silico* putative interaction of the flavonoid with an L-type calcium channel and NOS. **6-HOF** docking results against NOS and L-type calcium channels showed that, in both cases, it may bind with lower binding affinity than the ligands used as reference. For NOS, **6-HOF** had a binding affinity energy of -8.9 ± 0.0 kcal/mol versus -7.36 ± 0.05 kcal/mol for 3-bromo-7-nitroindazole (2.66 ± 0.49 Å against crystallographic pose). NOS docking with **6-HOF** revealed that it binds in nearly in the same position as 3-bromo-7-nitroindazole utilizing similar interactions. The lower binding-affinity energy of **6-HOF** might be attributable to better stacking interaction with TRP447 and to a large van der Waals-interaction surface when compared to 3-bromo-7-nitroindazole, as noted in the interaction map (Figure 4). 3-Bromo-7-nitroindazole and **6-HOF** interacted with nearly the same residues. However, **6-HOF** underwent interactions with five additional residues (VAL449, PRO450, PRO451, GLU361, and MET355), which 3-bromo-7-nitroindazole cannot reach because of its shorter molecular size. Calcium channel results demonstrated that **6-HOF** binding-affinity energy was -7.78 ± 0.0 kcal/mol versus -5.75 ± 0.31 kcal/mol for Nifedipine. Thus, **6-HOF** possesses a better affinity for the L-type calcium channel; as it can be seen in Figure 5 (upper right), both ligands (Nifedipine and **6-HOF**) fit at the same binding pocket with the same interactions. However, **6-HOF**, because of its lack of substituents, can fit deeper into the binding pocket having more interactions with residues. As with eNOS, **6-HOF** interacted with the same residues as Nifedipine but with three additional residues (CYS14, GLY49, and CYS46), because of its larger size. Taken together, the docking results indicate that **6-HOF** blocked ROC and VOC, that it could interact with

the binding pockets that access the catalytic site, and that this interaction might activate eNOS.

3.2 | *In vivo* antiasthmatic effect of 6-HOF by anti-allergic action

Taking in account previous reports in which the efficacy of flavonoids as experimental antiasthmatic drugs was described (Goszcz et al.,

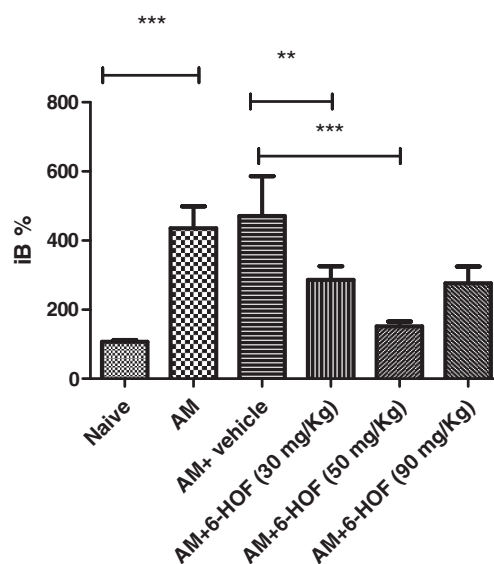


FIGURE 6 Anti-allergic effect of 6-hydroxyflavone on aerosolized ovalbumin-induced acute bronchial obstruction in guinea pigs. The results are presented as the mean $\pm$ the standard error of the mean, $n = 7$ (** $p < .01$, *** $p < .001$). iB = index of bronchial obstruction, AM = asthma model

Histology

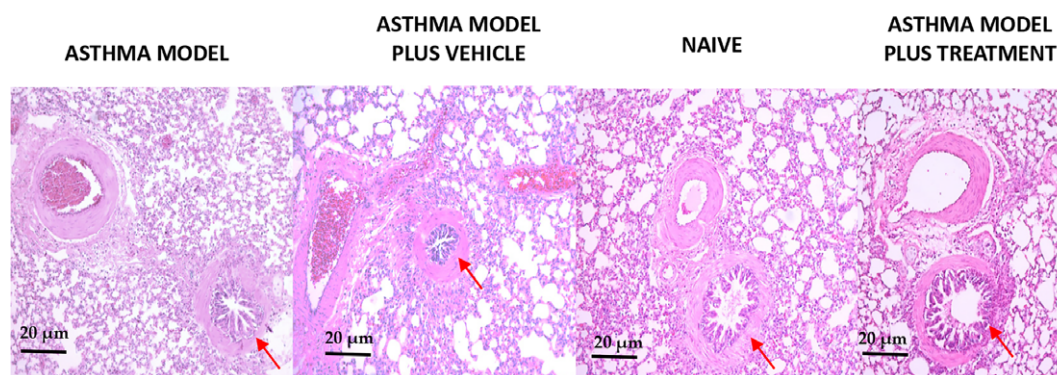


FIGURE 7 Comparative micrographs of lung sections showing bronchioles of a guinea pig asthma model after treatment with 6-hydroxyflavone or vehicle

TABLE 1 Dose used, body weight, and animal mortality in the 6-HOF acute toxicity study

Dose (mg/kg)	Animals per group	Change in body weight	Mortality
5	3	No	0/3
50	3	No	0/3
300	3	No	0/3
2,000	3	No	0/3

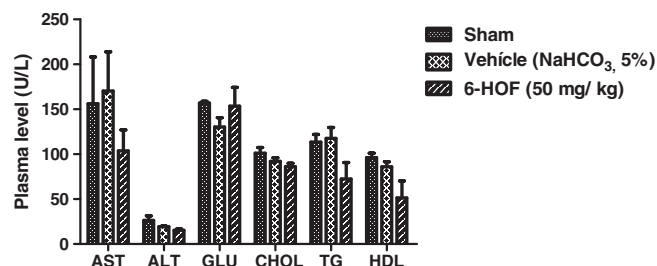


FIGURE 8 Blood transaminases, glucose, and lipid profiles determined in animals treated daily with 6-hydroxyflavone (50 mg/kg) for 28 days, vehicle, and sham. Each bar represents the mean $\pm$ the standard error of the mean of at least three animals compared with the sham and vehicle groups ($*p < .05$). AST = aspartate aminotransferase; ALT = alanine aminotransferase; GLU = glucose; CHOL = cholesterol; TG = triglycerides; HDL = high-density lipoprotein

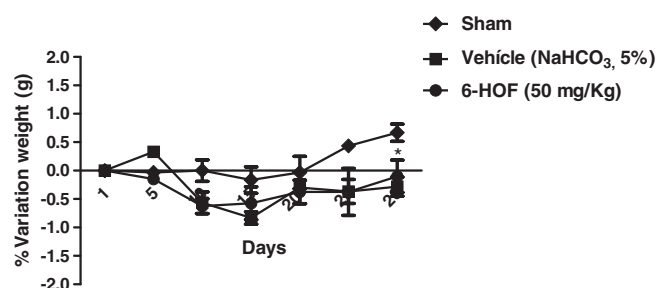


FIGURE 9 Body weight in animals daily treated and untreated with 6-hydroxyflavone (50 mg/kg) for 28 days. Each bar represents the mean $\pm$ the standard error of the mean, $n = 6$ ($*p < .05$)

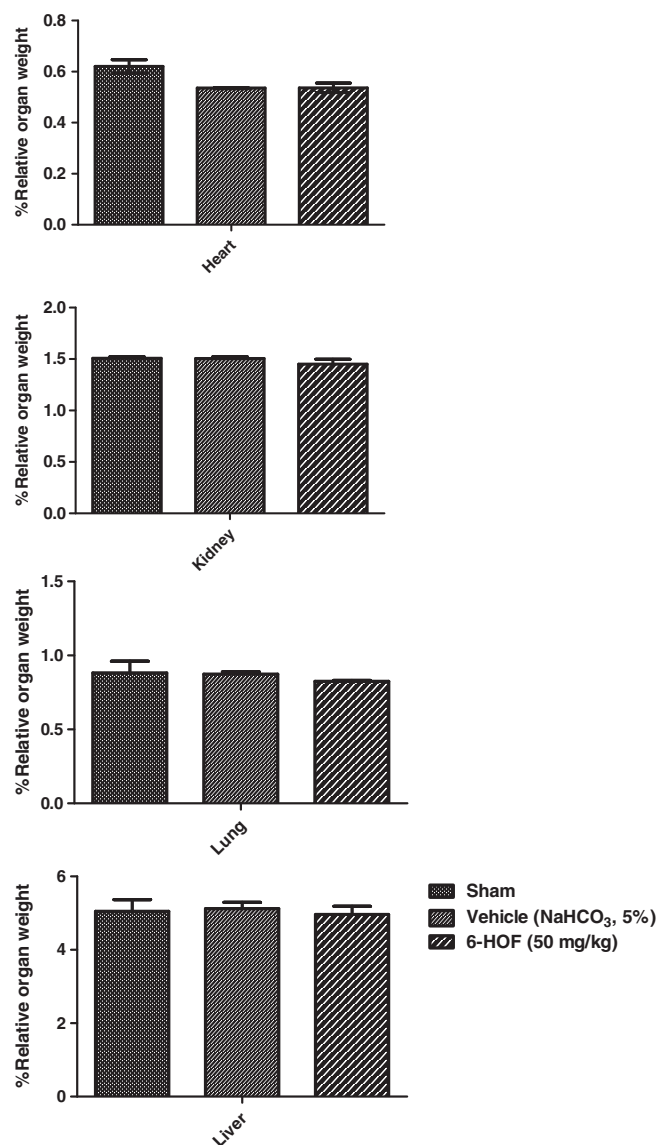


FIGURE 10 Relative weight of heart, kidney, lung, and liver in animals daily treated and untreated with 6-hydroxyflavone (50 mg/kg) for 28 days. Each bar represents the mean $\pm$ the standard error of the mean of at least three animals compared with sham

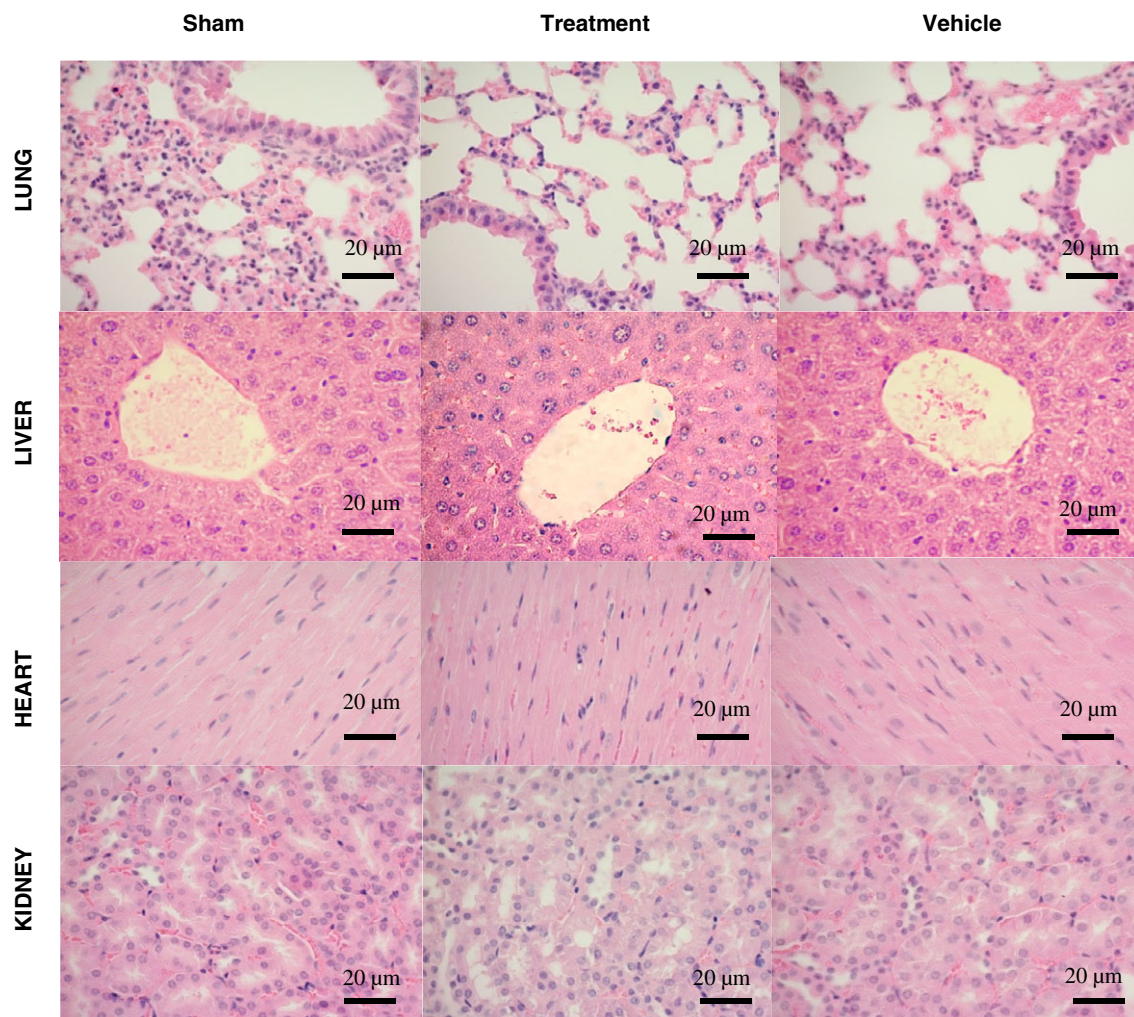
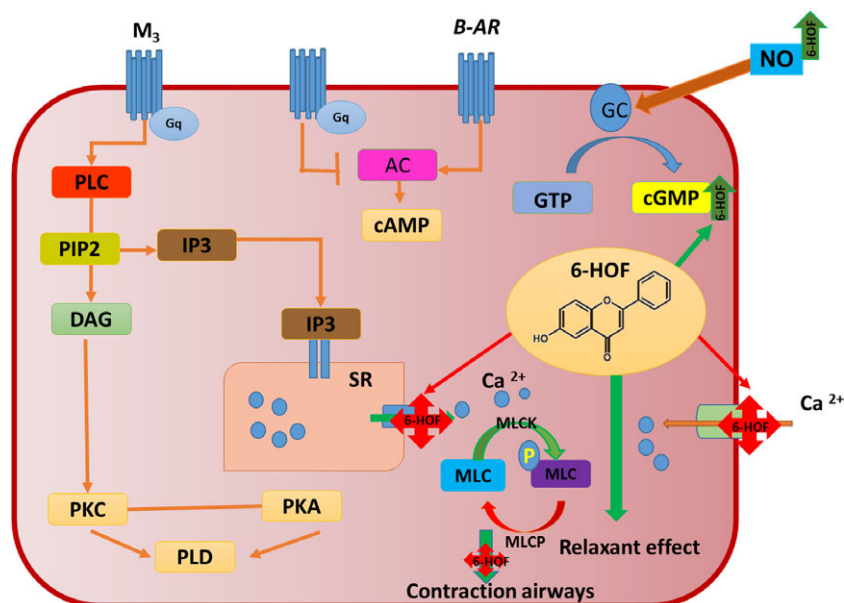


FIGURE 11 Histology of lung, liver, heart, and kidney from mice treated for 28 days with repeated administration of 6-hydroxyflavone (**6-HOF**) in toxicity-dosage study. Representative micrographs of each group (40×X): treated group with **6-HOF** (50 mg/kg), vehicle (solution of NaHCO₃ 5%), and sham. There are no histological abnormalities among the groups (H&E-stained sections)

2017; Tanaka & Takahashi, 2013; Wang et al., 2018) and the relaxant effect that was exhibited by **6-HOF** (Flores-Flores et al., 2018), we decided to explore the potential antiasthmatic effect of this flavonoid in allergic OVA-induced bronchoconstriction in a guinea pig model. Animals exposed to OVA three times for 15 days (at Days 0, 8, and 15) were treated with **6-HOF** at 30, 50, and 90 mg/kg i.p. 45 min before the last OVA challenge. After that, bronchoconstriction was measured. As observed in Figure 6, **6-HOF** decreased OVA-induced bronchoconstriction in a dose-dependent manner (30 and 50 mg/kg); however, the effect at 90 mg/kg was less efficient than at 50 mg/kg. Histological slides of lungs (H&E) from guinea pigs treated and untreated with **6-HOF** (50 mg/kg) were analyzed (Figure 7). **6-HOF** produced a significant opening of bronchioles compared with that of the naïve and vehicle groups, suggesting very important antiasthmatic action. The decrease of bronchoconstriction induced by the flavonoid could be related with inhibiting the degranulation of the mast cells and/or with lowering IgE, IL-4, IL-5, and IL-13 levels, as reported for other flavonoids (Tanaka & Takahashi, 2013). Further experiments are necessary to determine the immunomodulatory action of **6-HOF**.

3.3 | In vivo acute and sub-chronic toxicological studies

For preclinical studies in the development of potential drugs, it is necessary to determine their efficacy and the safety involved in their use. Thus, we decided to evaluate toxicity in experimental animals to establish the safety or potential toxicity of **6-HOF**. Flavonoids currently entertain a high impact on several biological systems, which depends on their chemical structure, because of various substituents such as glycosides, esters, amides, and hydroxyls, among others, which modulate the polarity, toxicity, and the extracellular and/or intracellular actions of these compounds (Flores-Flores et al., 2018; Goszcz et al., 2017; Harborne & Williams, 2000). Thus, OECD Guideline 425 with modifications was utilized to determine the acute toxicity of **6-HOF**. Table 1 reveals that treatment with different doses of the flavonoid (5, 50, 300, and 2,000 mg/kg) did neither cause death in the animals nor a change in their body weight. Also, the animals were observed carefully during the first 4 hr and exhibited neither signs of toxicity such as tremors, seizures, salivation, piloerection, diarrhea, and lethargy nor in behavioral and physical changes (skin, coat, eyes,



SCHEME 1 Representation of the signal transduction pathways involved in the regulation of airway smooth muscle contraction and the potential interaction of the 6-hydroxyflavone (**6-HOF**)-induced relaxant effect by calcium-channel blockade and enhanced production of nitric oxide and cyclic guanosine monophosphate. Green arrows represent pathways activated by **6-HOF** and red arrows and crosses show the **6-HOF** blockade of calcium channels, avoiding airway smooth muscle contraction. AC = adenylate cyclase; cAMP = cyclic adenosine monophosphate; SR = sarcoplasmic reticulum; PIP₂ = (4,5)-bis phosphate to generate second messengers; IP₃ = inositol trisphosphate; DAG = diacylglycerol

or mucous membranes). The animals did not show signs of toxicity during the 14 days following administration. The LD₅₀ of the **6-HOF** was estimated to be greater than 2,000 mg/kg, which allowed classifying it in Category 5 as a nontoxic compound based on OECD Guideline 425, with relatively low acute toxicity, but that, under certain circumstances, it may pose a hazard to vulnerable populations. Compounds classified in this category are less toxic than others.

However, after 28 days of treatment with **6-HOF** (50 mg/kg) by intragastric route, the mice exhibited any significant alterations neither in blood-glucose and lipid profiles nor in liver-marker enzymes (alanine aminotransferase and aspartate aminotransferase) (Figure 8), indicating that metabolism and liver function was not altered. Moreover, no deaths or symptoms of toxicity were documented during the 28 days of treatment by oral route with **6-HOF**. The animals did not show any changes in general behavioral or in other physiological activities, such as pain sensitivity and locomotion. A similar absence of toxicity was observed regarding food and water consumption; also, no change in body weight in the treated animals was observed (Figure 9). Nevertheless, at the end of the sub-chronic study, sham animals gained weight compared to those treated with the extract or the vehicle, probably because of the handling of these groups during **6-HOF** administration. Finally, there was no change in the relative weight of the organs dissected out (heart, kidney, lung, and liver) after 28 days of daily treatments with the flavonoid (Figure 10).

Moreover, histological analysis revealed that daily administration of **6-HOF** during the sub-chronic period did not cause pathological alterations in heart, kidney, lung, and liver when compared with normal and sham control mice. Histological sections of diverse tissues from treated or control animals did not demonstrate necrosis or inflammation (Figure 11), and the cyto-architecture was well

preserved in all studied tissues. Oral, sub-chronic treatment with 50 mg/kg of the flavonoid during 28 days did not alter blood transaminases, suggesting that the sample test did not produce toxicity. Furthermore, histopathological analysis of the lung, liver, heart, and kidney indicates that all of these were normal that, on their being well intact, correlated with biochemical data. Thus, the results of the present study contributed solid evidence in terms of the safety and pharmacological efficacy of **6-HOF** as a potential antiasthmatic drug and also demonstrated its nontoxic activities at the physiologic and cellular levels (Ávila-Villareal et al., 2016; Cassani et al., 2015; Hernández-Vázquez et al., 2017).

4 | CONCLUSION

These experiments suggest that **6-HOF** possesses significant relaxant activity through calcium channel blockade on rat trachea, which interferes with the contraction mechanism of smooth muscle cells in the airways as main mode of action. It appears that NO and cGMP production are involved in the relaxant effect (Scheme 1). Furthermore, based on the pharmacological and safety evidence, we propose this flavonoid as a potential lead for the development of novel therapeutic agents for the treatment of asthma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Idea and design of experiments: S.E.S., B.B.P., and R.V.M. ex vivo functional experiments: A.F.F., S.E.S., and R.V.M. Docking studies: C.M.P. and S.E.S. Antiasthmatic studies: A.F.F., B.B.P., and L.M.F. Toxicological profiles: A.F.F., S.E.S., and R.H.P. Blood biochemical profiles: A.F.F. and S.G.J. Analyzed data: A.F.F., S.E.S., J.C.R.L., and B.B.P. Contributed to the writing of the manuscript: S.E.S. and A.F.F. All authors have provided several corrections for comments on the manuscript and approval of the final version of the manuscript.

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