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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Relaxant effect of structurally related flavonoids on isolated tracheal rat rings: a SAR study

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Abstract In the search for potential new antiasthmatic drugs, the ex vivo relaxing effect and structural activity relationship (SAR) studies of a series of ten structurally related flavonoids were established. Also, glycosylated and prenylated flavonoids were included in this study in order to explore their relaxant effect. All flavonoids studied showed a significant relaxant effect on the contraction induced by carbachol (CCh) 1 μM on tracheal rat rings, with the exception of isoprenylated and glycosylated derivatives. Results indicated that the flavone scaffold or their 6-substituted or 7-substituted positions, exhibited best relaxant activity, being flavone (**1**), 6-hydroxyflavone (**2**), 6-aminoflavone (**3**), and 7-hydroxyflavone (**6**) the most active compounds, even more than the positive control (Theophylline). On the other hand, SAR analysis suggested that the presence of hydrogen-bond donor and acceptor substituents in position 6- or 7- in the flavone core, or the 2 and 3 double bond, and the increasing presence of hydroxyl groups in positions 4′-, 5′-, 6′-, and 6- could enhance the ex vivo relaxant effect of the flavonoids studied. Derived from SAR analysis, a pharmacophore mapping was proposed, taking into account the structure of 6-hydroxyflavone, in order to have the scaffold to show the best ex vivo relaxant activity.

Keywords Antiasthmatic drug · Flavonoids · 6-hydroxyflavone · Relaxant effect

Introduction

The Global Initiative for Asthma (GINA) defines it as a chronic inflammatory disorder of the airways. Chronically inflamed airways are hyperresponsive; they become obstructed and airflow is limited by reversed bronchoconstriction, mucus plugs, and increased inflammation when the airways are exposed to various risk factors such as environmental (GINA 2017). Currently, asthma affects approximately 300 million persons worldwide, and in Mexico, 4940 deaths were caused by asthma (GINA 2017; Shrimanker and Pavord 2017). Symptoms of asthma are treated with anti-inflammatory and bronchodilator inhaled agents (GINA 2017); however, with these treatments there are many adverse effects and/or symptoms that are not well controlled (Land and Wang 2017). In this context, plants especially those with ethnopharmacological uses have been the primary source for early drug discovery. Furthermore, many bioactive compounds have been isolated from these or have served as a prototypes to develop new molecules of therapeutic interest (Taur and Patil 2011; Cragg and Newman 2013). In recent years, there has been increasing interest in the study of flavonoids, which are plant

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polyphenolic-compound secondary metabolites, and more than 6000 structures have been reported. Flavonoids have exhibited important pharmacological activities, such as antioxidant, antiallergic, anti-inflammatory, immunomodulatory, antiasthmatic (Harborne and Williams 2000; Tanaka and Takahashi 2013), and relaxant agent of vascular smooth muscle (Ajay et al. 2003; Torres-Piedra et al. 2011), among others. Due to these perceived beneficial activities and their low toxicity, along with the growing interest in alternative medicine in disease prevention and treatment, flavonoids have an important impact on the future development of antiasthmatic drugs (Mali and Dhake 2011). Thus, the aim of the current study was to evaluate the relaxant effect of structurally related flavonoids on tracheal rat rings and develop a qualitative structure–activity relationship (SAR) in order to offer a preliminary insight into the impact of the pharmacologic activity of these molecules.

Materials and methods

Chemicals

All chemicals were ACS grade; flavone (**1**), 6-hydroxyflavone (**2**), 6-aminoflavone (**3**), 6-methoxyflavone (**4**), 6-chloroflavone (**5**), 7-hydroxyflavone (**6**), 3-hydroxyflavone (**7**), chrysin (**8**), quercetin (**9**), (±)-naringenin (**10**), hesperidin (**12**), naringin (**13**), and rutin (**14**) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dihydrospinochalcone-A (**11**) was isolated from *Lonchocarpus xuul* Lundell (Escalante-Erosa et al. 2012; Yam and Peña-Rodríguez 2009). For the ex vivo experiments, all compounds were dissolved with dimethyl sulfoxide (DMSO) (1%) and then diluted with distilled water.

Animals

All of the animal procedures were conducted according to the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999), and in compliance with International Guidelines on Care and Use of Laboratory Animals. Wistar rats weighing between 200 and 300 g were fed a standard rodent diet ad libitum with free access to water.

Pharmacological studies

Isolation of thoracic tracheal rings

Wistar rats were killed by an overdose of ethyl ether. The trachea was removed and placed in a Petri dish containing Krebs solution with the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; EDTA, 0.026; glucose, 11.1, pH 7.4, maintained at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2. This was carefully cleaned of adherent fat, connective tissue, and mucus and then cut into rings measuring 3 mm in width and 7 mm in length during the experiments. Each ring was placed in an incubation chamber, tissues were subjected to 2 g tension, and isometric tension was measured and recorded utilizing Grass-FT03 force transducers (Astromed®, West Warwick, RI, USA) connected to a MP100 analyzer (BIOPAC® Instruments, Santa Barbara, CA, USA). The tissues were equilibrated for at least 1 h prior to the addition of the drugs and during this time, the buffer solution was renewed every 20 min before starting the experiments.

Pharmacological relaxant effect

After a 30-min washout period, the tracheal rings were precontracted with carbachol (CCh) (1 μM) twice at 30-min intervals and washed with Krebs solution. To determine the relaxant effect, after stabilization, the tissues were precontracted again with submaximal contraction of CCh (1 μM) and, once the plateau was attained, concentration-response curves of the flavonoids (**1–14**), positive control (Theophylline), and vehicle (DMSO 1%) were obtained by adding cumulative concentrations of the test samples to the bath (Estrada-Soto et al. 2012).

SAR analysis

The basic structure used was flavone (**1**) and the relaxant activity of nine flavonoids (**2–10**) was employed for qualitative study; the activity was correlated with its chemical modifications (Table 1, Scheme 1).

Semisynthesis of 6-methoxyflavone (4)

6-Hydroxyflavone (8.39 mmol) was dissolved in acetone (2 mL), potassium hydroxide (20.89 mmol), as base and water (117 μL) were added to reaction mixture, which turned into a yellow solid; then, drop-wise dimethylsulfate (159 μL) was added and the mixture was heated at 40 °C for 24 h (monitored by TLC). The reaction mixture was washed with ice water (2 mL) and filtered under vacuum. Finally, the solid product (146.1 mg) was washed with NaOH (5%), and the mixture was shaken vigorously and filtered in vacuo to obtain 146.1 mg (yield of 63.8%). Compound was recrystallized from ethanol, with a melting point of 162–163 °C.

Structural characterization

After 6-methoxyflavone (**4**) (Scheme 1) was synthesized, analysis of ^{1}H-NMR spectrum was recorded that allowed to

Table 1 Half maximal effect concentration (EC_{50}) Maximal effect (E_{max}), relative potency and some physicochemical properties of flavonoids evaluated on rat tracheal smooth muscle contracted by carbachol 1 µM

Compound	EC_{50} (µM)	E_{max} (%)	Relative potency	PSA (A°)	Log *P*
Theophylline	152.85 ± 1.76	99.41 ± 1.27	1	94.40	−1.097
Flavone (**1**)	113.36 ± 4.42	95.73 ± 1.45	1.35	30.21	3.737
6-hydroxyflavone (**2**)	84.14 ± 4.48	92.47 ± 1.7	1.82	50.43	3.234
6-aminoflavone (**3**)	134.31 ± 4.3	91.98 ± 2.86	1.14	56.23	2.789
6-methoxyflavone (**4**)	184.96 ± 1.5	53.65 ± 5.2	0.83	39.44	3.77
6-chloroflavone (**5**)	NA	39.92 ± 2.85	NA	30.21	4.391
7-hydroxyflavone (**6**)	134.38 ± 0.75	89.08 ± 2.59	1.14	50.43	3.234
3-hydroxyflavone (**7**)	288.21 ± 5.6	69.11 ± 5.43	0.53	50.43	3.446
Chrysin (**8**)	193.36 ± 3.95	62.57 ± 3.34	0.79	70.66	2.94
Quercetin (**9**)	198.66 ± 1.35	92.4 ± 3.83	0.76	131.35	1.683
(±)-naringenin (**10**)	264.91 ± 1.88	60.78 ± 3.34	0.58	86.98	2.117
Dihydroespinochalcone A (**11**)	NA	40.51 ± 1.51	NA	ND	ND
Hesperidin (**12**)	NA	38.82 ± 3.34	NA	ND	ND
Naringin (**13**)	NA	34.98 ± 5.69	NA	ND	ND
Rutin (**14**)	NA	18.56 ± 3.5	NA	ND	ND

NA no active, *ND* not determined

corroborate the chemical structure of **4**, through the comparison of the experimental spectroscopic data obtained with the data reported in the literature (Sunhee et al. 2008) (Suplementary Material, Table 2).

Computational methods

Polar surface area (PSA)

High oral bioavailability is an important factor for the development of bioactive molecules as therapeutic agents. Passive intestinal absorption, reduced molecular flexibility (measured by the number of rotatable bonds), low PSA, or total hydrogen bond count (sum of donors and acceptors) are found to be important predictors of good oral bioavailability (Cragg and Newman 2013). Thus, MiLogP (octanol/water) and PSA were calculated by the method developed by the Molinspiration site (http://www.molinspiration.com/cgi-bin/properties) as a sum of contributions from fragments and correction factors. PSA is a very useful parameter for the prediction of drug transport properties and is defined as the sum of surfaces of polar atoms (usually oxygens, nitrogens, and attached hydrogens) in a molecule. This parameter has been shown to correlate very well with human intestinal absorption, Caco-2 monolayer permeability, and Blood–Brain Barrier penetration (Ertl et al. 2000).

Pharmacophore building

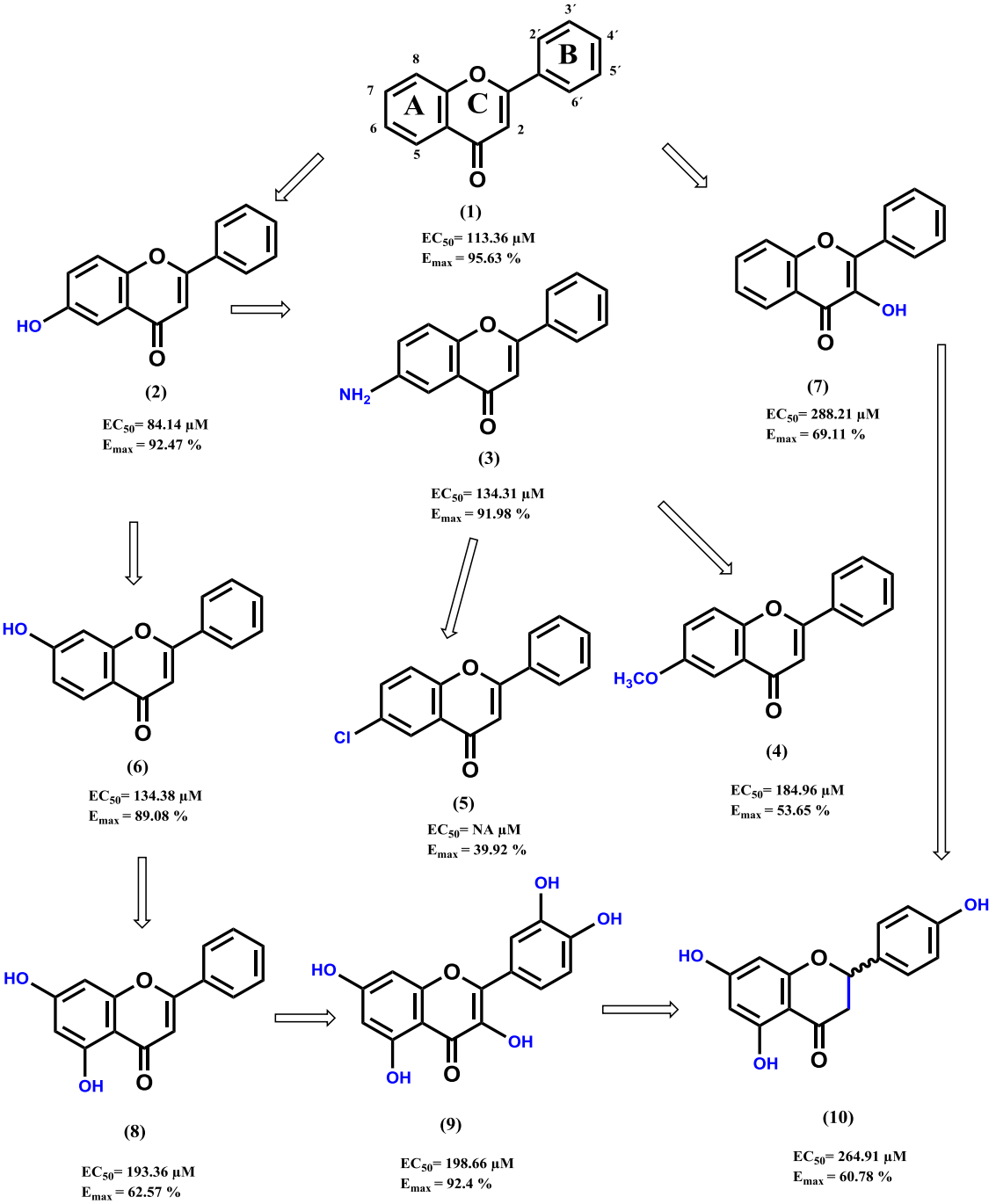
A pharmacophore is a molecular fragment that bears the essential features responsible for the biological activity of a drug based on key molecular features, such as steric and electrostatic characteristics and/or hydrogen-bonding capacity. Based on the latter, the construction of a pharmacophore from the comparison of relaxant effect related with structural flavonoid analogs **1–10** allowed to know the minimal features required to build a potential antiasthmatic flavonoid using the Discovery Studio software program (Dassault Systèmes BIOVIA 2016). This program has been widely used to create pharmacophore hypotheses, generating three-dimensional queries for virtual or experimental screening (Hidalgo-Figueroa et al. 2017; Gogoi et al. 2017).

Statistical analysis

Pharmacological data were analyzed by two-way analysis of variance, and $p < 0.05$ was considered statistically significant. All values are expressed as the mean ± standard error of the mean. Concentration response curves were plotted and the experimental data obtained were adjusted by means of a nonlinear curve-fitting program (ORIGIN 8.0).

Result and Discussion

All flavonoids studied were selected based on five aspects: (a) using the flavone moiety as starting scaffold; (b) including hydroxyl groups as mono-substituents in positions -3, -6, and -7 (A and C rings); (c) replacing hydrogen bond donor/acceptor for other functional groups (A ring); (d) increasing the number of the hydroxyl groups to select poly-hydroxylated flavonoids (A, B, and C rings); and (e) absence or presence of double bond between positions 2



Scheme 1 Structure-activity relationships of flavonoids **1–10**. *NA* no active

and 3 (Mastuda et al. 2002) (Scheme 1). Also, we included three glycosylated flavonoids and one prenylated chalcone (Escalante-Erosa et al. 2012; Yam and Peña-Rodríguez 2009) in the relaxant study (Table 1); however, these were not taken in account for SAR analysis.

Thus, selected compounds were evaluated [0.1–500 µM] on the contraction induced by CCh (1 µM, acting as muscarinic cholinergic agonist) in the tracheal rat rings, and Theophylline (a non-selective phosphodiesterase inhibitor) was utilized as positive control. Table 1 presents the values of one-half maximal effective concentration (EC_{50}), maximum effect (E_{max}), as well as calculated relative potency. As observed, nearly all flavonoids exhibited a significant relaxant effect, with the exception of 6-chloroflavone (**5**), prenylated chalcone (**11**), and glycosylated flavonoids (**12-14**).

The most active compound was the mono-substituted 6-hydroxyflavone (**2**), followed by 6-aminoflavone (**3**) and

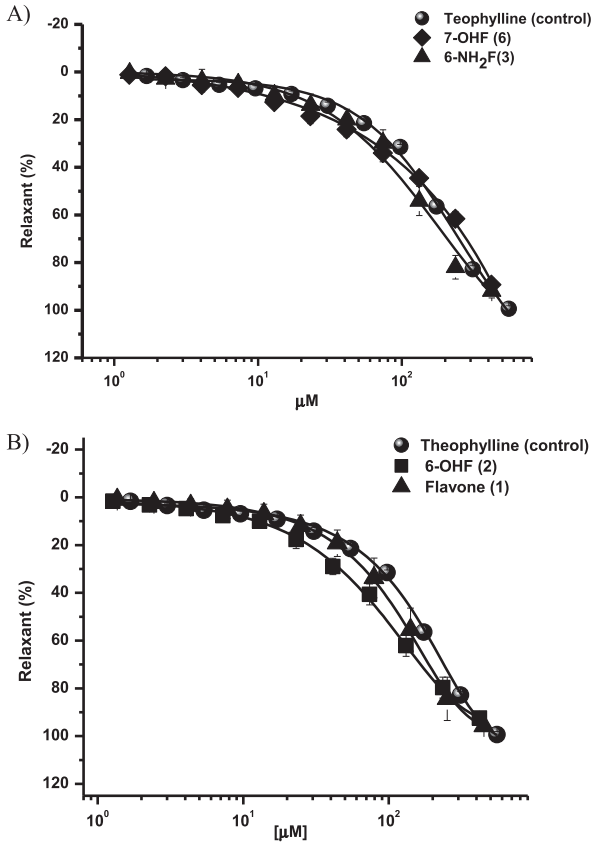


Fig. 1 Concentration–response curves of the relaxant effect showed by **a** 6-aminoflavone (**3**) comparing with 7-hydroxiflavone (**6**), **b** flavone (**1**) comparing with 6-hydroxyflavone (**2**) on the contraction induced by carbachol on the isolated rat tracheal rings. Each point represents the mean ± S.E.M. of six experiments. In some points of the figure, the S.E.M. does not appear for being very close to the symbols

7-hydroxyflavone (**6**) (Figs. 1a and 1b). Also, flavone (**1**) demonstrated similar potency and efficacy as those of compounds **3** and **6**. Flavonoids **1–3** and **6** showed a concentration-dependent relaxant effect and nearly 100% efficacy, and were more potent than positive control ($p < 0.05$).

On the other hand, SAR analysis was carried out using the experimental EC_{50} and E_{max} values from the relaxant effect shown for the flavonoids **1–10** studied. Scheme 1 displayed the qualitative SAR studies conducted, with their findings suggesting a common scaffold (flavone), and mono-substitution with hydroxyl group at position-6 significantly increased the potency and efficacy observed in the flavone. Also, replacement with several monovalent isosteres (–NH_2 or –OMe) at the same position lead to weakness in efficacy and potency and, in some cases, caused complete loss of activity (–Cl). On the other hand, the presence of the hydroxyl group at position -3 reduced potency and efficacy; however, when –OH is located at -7 and/or -5 or only at -7 positions, a slight reduction in potency is causes (7-hydroxyflavone and 5,7-dihydroxyflavone, respectively). The introduction of –OH on the B ring (-3′ or -3′ and -4′ positions) did not cause any changes in biological activity; moreover, it appears that all flavonoids must conserve the ketone group as H-bond acceptor, and the presence of the double bond between 2 and 3 is necessary, since when it is removed, activity drastically diminished (such as in the case of naringenin). It is noteworthy that the presence of a carbohydrate derivative as substituent in different positions induced total loss of relaxant activity. Derived from SAR analysis, a pharmacophore model was constructed and has been developed to explain the relaxant activity. The hypothetic features (meshed spheres) are depicted in Fig. 2. Thus, polar groups (–OH) in 6-position of ring A, and in 3′- and 4′-position of ring B, enhanced relaxant activity. Also, to preserve potency, aromatic rings A and B are necessary.

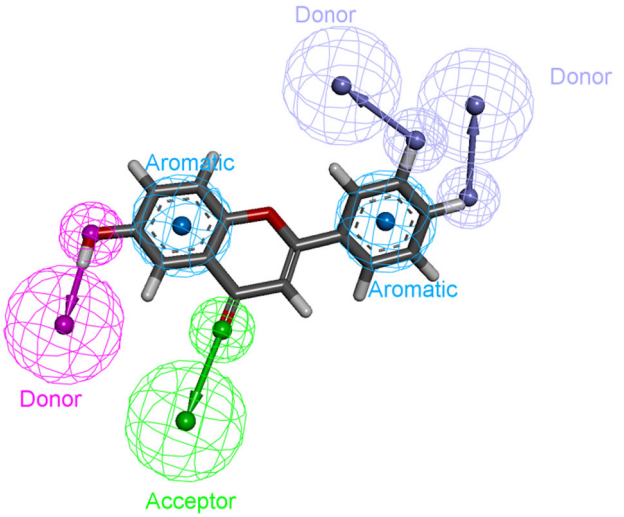


Fig. 2 The hypothetic pharmacophore pattern with 6-hidroxyflavone fitting in four points

Table 1 also illustrates the surface polar area that influences both the interaction and the absorption properties of flavonoids studied. Based on the results, it was observed that only five compounds fall within the accepted parameters of PSA (90–140 $Å^2$), which, in agreement with Veber rules for good oral bioavailability in rats (Remko et al. 2006) and Log *P* between 2.7 and 5 (Kerns and Di 2015), compounds **1–3**, **6**, and **7** showed the best characteristic for absorption and interactions, which clearly is in agreement with best relaxant activity determined.

In conclusion

The effect appears to be variable depending on the substituent in position -6; thus, when 6-methoxyflavone and chloroflavone were evaluated, the activity drastically reduced more than the activity shown by 6-hydroxyflavone

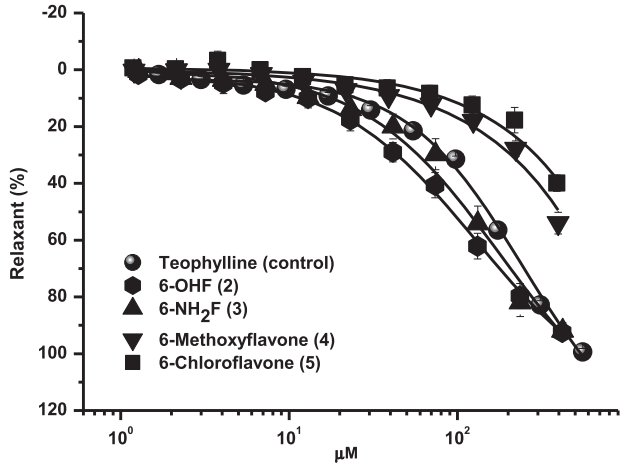


Fig. 3 Relaxant concentration–response curves of 6-hydroxyflavone (**2**), comparing with 6-aminoflavone (**3**) and 6-methoxyflavone (**4**) on the carbachol-contracted isolated rat tracheal rings. Each point represents the mean ± S.E.M. of five experiments

(**2**) and 6-aminoflavone (**3**), which demonstrated a significant concentration-dependent relaxant effect (Fig. 3). These latter results indicate that the flavone scaffold is necessary to present hydrogen-bond donors or acceptors but, if both proton donors and acceptors exist (such as the –OH group) in position -6, activity is improved. Also, the presence of the hydroxyl group in the -7 position also preserves relaxant activity in tracheal rat rings. In this context, 6-hydroxyflavone (**2**) exhibited significant relaxant activity in contractions induced by CCh in tracheal rat rings, which represents a potential prototype for future preclinical studies in order to develop a novel antiasthmatic drug. Also, based on the SAR study, we could design and semi-synthesize compounds employing the proposed pharmacophore.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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