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Central nervous system depressant action of flavonoid glycosides

Sebastián P. Fernández [a,b], Cristina Wasowski [a], Leonardo M. Loscalzo [a], Renee E. Granger [b], Graham A.R. Johnston [b], Alejandro C. Paladini [a], Mariel Marder [a,*]

[a] Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Junín 956 (C1113AAD), Buenos Aires, Argentina
[b] Adrien Albert Laboratory of Medicinal Chemistry, Department of Pharmacology, Faculty of Medicine, University of Sydney, Sydney, NSW 2006, Australia

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Abstract

The pharmacological effects on the central nervous system (CNS) of a range of available flavonoid glycosides were explored and compared to those of the glycosides 2*S*-hesperidin and linarin, recently isolated from valeriana.

The glycosides 2*S*-neohesperidin, 2*S*-naringin, diosmin, gossipyn and rutin exerted a depressant action on the CNS of mice following i.p. injection, similar to that found with 2*S*-hesperidin and linarin. We demonstrate in this work that these behavioural actions, as measured in the hole board, thiopental induced sleeping time and locomotor activity tests, are unlikely to involve a direct action on γ-aminobutyric acid type A ($GABA_A$) receptors. The corresponding aglycones were inactive, pointing to the importance of the sugar moieties in the glycosides in their CNS depressant action following systemic administration.

The pharmacological properties of the flavonoid glycosides studied here, in addition to our previous results with hesperidin and linarin, opens a promising new avenue of research in the field.

Keywords: Flavonoid glycosides; Hesperidin; $GABA_A$; Sedation; Sleep-enhancing action

1. Introduction

Flavonoids are low molecular weight compounds present in all higher plants. To date, more than 5000 structurally distinct flavonoids have been described. The diversity in their chemical structure confers them a wide range of biological activities. In plants, their function seems to be linked to protection against ultraviolet radiation, microbial invasion and both insect and mammalian herbivores.

Their actions in humans have been the subject of extensive research and they have been described to possess numerous biological activities such us antioxidant, anti-inflammatory, oestrogenic, cytotoxic antitumoral, antiviral and others (Harborne and Williams, 2000). However, the number of clinical studies evaluating flavonoids in human therapy is limited and sometimes based only in epidemiological data, for example as promoters of cardiovascular health (Arts and Hollman, 2005; Fisher and Hollenberg, 2005). Perhaps the most relevant clinical data comes from their use in the treatment of bone loss, vascular diseases and cancer (Messina et al., 2004; Grendys et al., 2005; Katsenis, 2005; Van Veldhuizen et al., 2005).

Many flavonoids were found to be ligands for the γ-aminobutyric acid type A ($GABA_A$) receptors in the central nervous system (CNS); which led to the hypothesis that they act as benzodiazepine-like molecules. This is supported by their behavioral effects in animal models of anxiety, sedation and convulsion (Marder and Paladini, 2002; Johnston, 2005). Due to the increased knowledge of the diversity of $GABA_A$ receptor subtypes, the number of studies with cloned receptors of defined subunit composition has recently risen, and experiments with some natural and synthetic flavones and flavanones have shown that they can modulate γ-aminobutyric acid (GABA)-generated chloride currents, either positively or negatively (Goutman et al., 2003; Campbell et al., 2004; Kavvadias et al., 2004; Hall et al., 2005). However, none of these studies evaluated the action of flavonoid glycosides, despite the fact that they are the main forms of these compounds found in nature.

* Corresponding author. Tel.: +54 11 4962 5506; fax: +54 11 4962 5457.
E-mail address: mmarder@qb.ffyb.uba.ar (M. Marder).

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The only existing reports on the CNS action of flavonoid glycosides came from their isolation from plants used as tranquilizers. Kang et al. (2000) detected sedative action in mice of two flavonol glycosides, quercitrin and isoquercitrin, isolated from the flowers of *Albizzia julibrissin* Durazz. Du et al. (2002) found that goodyerin, a flavonol glycoside isolated from *Goodyera schlechtendaliana*, possesses sedative and anticonvulsant activities in mice and Datta et al. (2004) described that the flavonol glycoside quercetin-3-O-(6″-feruloyl)-β-D-galactopyranoside, isolated from the aerial parts of *Polygonum viscosum*, presented CNS depressant activity. In addition, a glycosilated flavanone, 2*S*-hesperidin (Table 1) (Marder et al., 2003) and a glycosilated flavone, linarin (Table 1) (Fernández et al., 2004), with sedative-hypnotic effects were identified by us in the roots and rhizomes of two valerian species. Relevant synergistic interactions were found when these two compounds were assayed in combination with other components of the same valerian extract, illuminating the existing vagueness behind the therapeutic actions of this plant (Fernández et al., 2004, 2005).

The aim of the present work was to study the pharmacological properties of a range of available flavonoid glycosides comparatively to those of 2*S*-hesperidin and linarin. It was found that 2*S*-neohesperidin, 2*S*-naringin, diosmin, gossipyn and rutin (Table 1) possess sedative and sleep-enhancing effects in mice following i.p. injection and we could demonstrate that these actions do not involve binding to the central $GABA_A$ receptor.

The present work signals flavonoid glycosides as a group of neuroactive compounds worth of attention in the search of new sedative and hypnotic drugs.

2. Materials and methods

2.1. Drug sources and injection procedure

The drugs used to perform the behavioral experiments are shown in Table 1 and were obtained as follows: gossypin, didymin, diosmin and isorhoifolin from Indofine Chemical Company, USA; eriocitrin, prunin, narirutin, eriodyctiol and

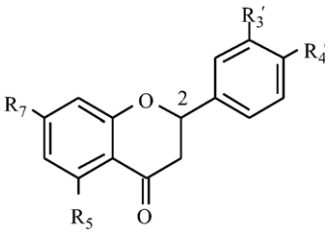
Table 1
Molecular structures of the investigated compounds

(A) Sugar moieties

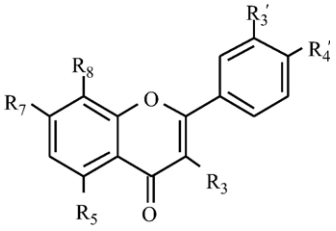
Glucose (Glc)
Neohesperidose: *O*-α-L Rhamnosyl-(1→2)-glucose (Nh)
Rutinose: *O*-α-L Rhamnosyl-(1→6)-glucose (Rt)

(B) Flavanones

Compound	R_5	R_7	$R_{3'}$	$R_{4'}$
Flavanone	H	H	H	H
Naringenin	OH	OH	H	OH
2*S*-Naringin	OH	*O*-β-Nh	H	OH
Prunin	OH	*O*-β-Glc	H	OH
Narirutin	OH	*O*-β-Rt	H	OH
Eriodictyol	OH	OH	OH	OH
Eriocitrin	OH	*O*-β-Rt	OH	OH
Didymin	OH	*O*-β-Rt	H	OCH_3
2*S*-Hesperetin	OH	OH	OH	OCH_3
2*S*-Hesperidin	OH	*O*-β-Rt	OH	OCH_3
2*S*-Neohesperidin	OH	*O*-β-Nh	OH	OCH_3



(C) Flavones and flavonols



Compound	R_3	R_5	R_7	R_8	$R_{3'}$	$R_{4'}$
Isorhoifolin	H	OH	*O*-β-Rt	H	H	OH
Linarin	H	OH	*O*-β-Rt	H	H	OCH_3
Diosmetin	H	OH	OH	H	OH	OCH_3
Diosmin	H	OH	*O*-β-Rt	H	OH	OCH_3
Quercetin	OH	OH	OH	H	OH	OH
Rutin	*O*-β-Rt	OH	OH	H	OH	OH
Gossypin	OH	OH	OH	*O*-β-Glc	OH	OH

flavanone from Extrasynthese, Genay, France; GABA, picrotoxin, picrotoxinin, quercetin, rutin and naringenin from Sigma-Aldrich, USA. 2*S*-Neohesperidin and 2*S*-naringin were isolated from small oranges aborted from the tree *Citrus hamlin*, and were kindly provided by Dr. Guillermo Ellenrieder of the Universidad Nacional de Salta, Argentina. Linarin and 2*S*-hesperidin were obtained by us (Marder et al., 2003; Fernández et al., 2004). 2*S*-Hesperetin was obtained by enzymatic hydrolysis according to Scaroni et al. (2002). Diosmetin was prepared by acid hydrolysis of diosmin as previously described (Marder et al., 2003). [^{3}H]t-Butyl-bicyclo-orthobenzoate ([^{3}H]TBOB) was purchased from Amersham Biosciences UK Ltd. (Amersham Place Little Chalfont, Buckinghamshire, UK) and [^{3}H]flunitrazepam from New England Nuclear, NEN. Diazepam was from Hoffmann-La Roche.

Chemical purity of 2*S*-neohesperidin, 2*S*-naringin, linarin, 2*S*-hesperidin and diosmetin were above 95%, estimated by us based on analytical HPLC experiments.

For injections the drugs were dissolved by the sequential addition of dimethylsulfoxide up to a final concentration of 5%, a solution of 0.25% Tween 80 up to a final concentration of 20% and saline to complete 100% volume. Sodium thiopental (Fada, Biochemie Gesellschaft m.b.H., Kundl/Tirol, Austria) was dissolved in saline. The rodents were i.p. injected 20 min before performing the pharmacological tests. The volume of i.p. injections was 0.15 ml/30 g of body weight. In each session, a control group receiving only vehicle was tested in parallel with those animals receiving drug treatment.

2.2. Animals

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays and adult male rats (200–300 g) Wistar strain for biochemical studies, both were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. Animals were housed in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h:12 h day/night cycle with light onset at 06:00 h. Housing, handling, and experimental procedures complied with the recommendations of the European Community guidelines for the use of experimental animals.

Behavioral experiments were conducted from 10:00 AM to 2:00 PM.

2.3. Behavioral studies

2.3.1. Sodium thiopental-induced sleeping time assay

A sub-hypnotic dose of sodium thiopental (35 mg/kg) was i.p. injected to mice 20 min after a similar injection of vehicle or the drug. Sleeping time was determined as the interval between the loss and the recovery of the righting reflex (Ferrini et al., 1974).

2.3.2. Hole board assay

This assay was conducted in a walled black Plexiglass arena (with nonreflecting surfaces) with a floor of 60 cm × 60 cm and 30 cm high walls, with four centered and equally spaced holes in the floor, 2 cm in diameter each. Each hole housed an infrared emitting diode and an infrared detector oriented along a diameter and perfectly aligned. Each of these pairs conforms a hole exploration sensor. The interruption of the infrared beam by an exploring mouse was analyzed by an ad hoc Visual Basic software running on a personal computer. The interruption and its duration were recorded. The detected interruption was ignored if the duration was 100 ms or less.

The mice were placed and released singly in the centre of the board, facing away from the observer. The number of holes explored and the duration of each of the explorations was shown in real time to the observer and at the end of the experiment (usually 5 min) all the information was automatically stored in a file for post experiment study. The number of mouse "rearings" was detected visually and recorded by the observer. After each trial the apparatus was wiped clean to remove traces of the previous assay. A decrease in the number of head-dips, the time spent head-dipping and/or the number of rearings reveals a sedative behavior (File and Pellow, 1985).

2.3.3. Locomotor activity assay

Spontaneous locomotion activity was measured after completing the hole board test, in a box made of Plexiglass, with a floor of 30 cm by 15 cm and 15 cm high walls. On the walls and along the longest axis of the box, 15 infrared emitting diodes and 15 infrared detectors were arranged in perfectly aligned pairs. Each of these pairs conforms a movement sensor. The distance of the sensors was 3 cm from the bottom of the box. The sensor interruptions measure the animal activity along a single axis (the long one). Exclusive OR logic was used to analyze the interruptions of successive sensors, therefore simultaneous interruptions of two or more successive sensors were counted as a single event. The interruption of a sensor and duration of this event were detected and recorded by a personal computer running a Visual Basic program developed specifically for this apparatus. The data was shown in real time to the operator and at the end of the experiment all the information was automatically stored in a file for post experiment study. The locomotor activity was expressed as total light beam counts per 5 min (Rabbani et al., 1995).

2.3.4. Picrotoxin blockade experiments

To explore the role of GABA system in the CNS depressant actions of these glycosides the pre-treatment with picrotoxin was used to attempt blocking the pharmacological action of 2*S*-hesperidin in the hole board test. 2*S*-Hesperidin, being the leader drug in the group, was selected for this assay. Picrotoxin and 2*S*-hesperidin were administered 30 and 20 min, respectively, prior to testing.

2.4. Biochemical experiments

2.4.1. Tissue preparation

Rats were humanely killed by decapitation, the brains rapidly removed and cerebral cortex dissected out. The cortex were homogenized in 10 volumes of 0.32 M sucrose at 0 °C and the

homogenate was centrifuged at 900×*g* for 10 min at 4 °C. Supernatant was decanted and centrifuged at 100,000×*g* for 30 min at 4 °C, the pellet was washed twice in 25 mM Tris–HCl buffer pH 7.4 for [^{3}H]flunitrazepam binding and seven times in 50 mM Tris–HCl plus 500 mM NaCl buffer pH 7.4 for [^{3}H] TBOB binding; in both cases homogenates were stored at −20 °C until used.

2.4.2. [^{3}H]flunitrazepam binding assay

On the day of the assay, membranes were thawed and duplicate samples containing 0.2–0.4 mg protein were suspended in a final volume of 1 ml of 25 mM Tris–HCl buffer, pH 7.4 in the presence of a solution of the sample assayed. The incubation was carried out at 4 °C for 60 min with 0.4 nM [^{3}H]flunitrazepam (81.8 Ci/mmol). Non specific binding was determined in parallel incubations in the presence of 10 μM flunitrazepam, and represented 5–15% of the total (Marder et al., 2003).

2.4.3. [^{3}H]TBOB binding assay

For the [^{3}H]TBOB binding assay, membranes were thawed and duplicate samples containing 0.1–0.2 mg protein were suspended in a final volume of 1 ml of 50 mM Tris–HCl plus 500 mM NaCl buffer pH 7.4 in the presence of a solution of the sample assayed. The incubation was carried out at room temperature for 90 min with 6 nM [^{3}H]TBOB (16 Ci/mmol). Specific binding was measured in well washed synaptosomal membranes with or without 0.3 μM GABA, since modulation of [^{3}H]TBOB binding by $GABA_A$ receptor ligands depends on the absence or presence of GABA (Ghiani et al., 1996; Rezai et al., 2003).

Non-specific binding was determined in parallel incubations in the presence of 100 μM picrotoxinin, and represented 30–35% of the total (Rezai et al., 2003).

After the incubations, for both radioligand binding assays, samples were rapidly diluted with 3 ml of ice-cold buffer, and immediately terminated by filtration under vacuum through Whatman GF/A glass-fiber filters, and then washed three times with 3 ml each of same buffer. Filters were counted after addition of Optiphase 'Hisafe' 3 (Wallac Company, Turku, Finland) liquid scintillation cocktail.

2.5. Electrophysiology experiments

These studies were performed according to the technique described by Granger et al. (2005). Briefly, *Xenopus laevis* oocytes were injected with cRNA (20 ng/50 nl) encoding $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ subunits in a ratio 1:1:2, respectively, to favour incorporation of the γ subunit. After 3 days of storage at 16 °C, oocytes were tested for $GABA_A$ receptor activity by two electrodes voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments), a MacLab 2e recorder (AD Instruments) and Chart version 3.5.2 program. Glass micropipettes were filled with 3 M KCl and resistance values were 0.5–2 MΩ. Holding potential was set to −60 mV and oocytes were continuously superfused with frog Ringer solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES). Flavonoids were added to the perfusion system dissolved in DMSO (final concentration of DMSO 0.6%) to test direct activation of the receptor; and together with 6 μM GABA to test modulation of GABA response.

2.6. Statistical analyses

When several treatments were compared, one-way analysis of variance (ANOVA) was used and post-hoc comparisons between individual treatments were made using Newman-Keuls multiple comparison test except for comparison of sleeping time, in this case Dunn's multiple comparison test was used after Kruskal-Wallis test (nonparametric analysis of variance).

Data from picrotoxin blockade experiment was analyzed by two-way ANOVA (pre-treatment vs. treatment) and post-hoc comparison was made using Bonferroni post-tests.

Binding data were logarithmically transformed and tested for significance using a linear regression fit, the *F* and *P* values shown are derived from this analysis. Provided the slope of the curve significantly deviated from zero, a nonlinear regression fit to one site competition model was performed using Prism 4.00, GraphPad software.

3. Results

3.1. Sleep-enhancing action of flavonoid glycosides and their aglycones

Table 2 shows the effects of the compounds on the sodium thiopental-induced sleeping time assay. The effects of 2*S*-hesperidin and linarin, taken from previous works, are shown for comparison. 2*S*-neohesperidin, 2*S*-naringin, diosmin and gossypin at 30 mg/kg and rutin at 20 mg/kg significantly increase the sleeping time. Rutin was the only glycoside derivative that exerted a significant action at 10 mg/kg. When compared with 2*S*-hesperidin and linarin none of the newly tested glycosides had a comparable activity. The strength of the flavonoid glycosides action in decreasing order is: 2*S*-hesperidin > linarin > rutin > diosmin ≅ 2*S*-neohesperidin > 2*S*-naringin > gossypin.

2*S*-Hesperetin, the aglycone of 2*S*-hesperidin and 2*S*-neohesperidin, and flavanone, the molecular nucleous of the flavanone glycosides, were unable to potentiate sodium thiopental induced sleep in mice at the dose tested.

Didymin, prunin, narirutin, eriodictyol, eriocitrin and isorhoifolin at doses of 10 mg/kg ($n=6$) showed no effect in the assay (Table 2). Higher doses were not attempted in part due to solubility issues but also to concentrate our efforts in the more interesting compounds.

3.2. Sedative action of flavonoid glycosides and their aglycones

The sedative action of these compounds, determined in the hole board test, are shown in Figs. 1 and 2. The effect of 2*S*-hesperidin at 5 mg/kg and linarin at 7 mg/kg are shown for

Table 2
Effect of the compounds on the thiopental induced sleeping time and spontaneous locomotor activities in mice

Sample	Dose (mg/kg)	Sleeping time		Locomotor activity	
		n	Median (interquartile range) (s)	*n*	Mean±S.E.M. (counts)
Vehicle	–	28	0 (0/15)	30	741.1±24.2
Flavonoid glycosides					
2*S*-Hesperidin	5	12	1710 (1050/1836)[c]	6	239.4±64.6[b]
Linarin	14	11	1320 (390/1800)[c]	–	ND
2*S*-Neohesperidin	10	13	0 (0/180)	8	638.6±31.0
	30	7	1020 (360/1350)[a]	9	415.8±34.2[b]
Diosmin	10	6	15 (0/528)	6	681.0±38.0
	30	9	1050 (765/1545)[c]	8	309.5±47.1[b]
Rutin	10	7	630 (369/1200)[a]	6	361.6±74.1[b]
	20	6	945 (293/1395)[b]	6	264.7±68.3[b]
2*S*-Naringin	10	6	30 (0/45)	6	714.0±46.9
	30	6	900 (510/1305)[a]	8	667.4±18.2
Gossypin	10	6	30 (0/45)	6	691.3±106.6
	30	6	780 (330/1665)[b]	9	649.0±35.0
Aglycones					
Flavanone	30	6	0 (0/0)	4	761.8±61.1
2*S*-Hesperetin	15	6	0 (0/0)	6	644.2±27.2
Diosmetin	15	–	ND	7	458.4±53.0[b]
Naringenin	15	–	ND	6	707.8±31.0
Quercetin	15	–	ND	7	671.7±56.2

Median (interquartile range) of sleeping time of mice measured in a sodium thiopental-induced sleep test and mean±S.E.M. of the spontaneous locomotor activity counts during 5 min test sessions 20 min after an i.p. injection of vehicle or the compounds. The sleeping time was measured as the time spent between disappearance and reappearance of righting reflex, the locomotor activity after completing the hole board test as described in Materials and methods; *n*=number of mice. ND: not determined.
Sleeping time: [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$, significantly different from vehicle; Dunn's multiple comparison test after Kruskal-Wallis test (nonparametric ANOVA).
Locomotor activity: [a]$P<0.05$, [b]$P<0.01$, significantly different from vehicle; Newman-Keuls multiple comparison test after ANOVA.

comparison. 2*S*-Neohesperidin and diosmin (Fig. 1A) significantly reduced all three parameters at 30 mg/kg (rearings and time head-dipping, $P<0.01$ for both compounds; head-dips, $P<0.01$ and $P<0.05$, respectively). 2*S*-neohesperidin also significantly decreased the number of rearings at 10 mg/kg ($P<0.05$). Rutin (Fig. 1B) significantly reduced the number of rearings at doses of 10 mg/kg and 20 mg/kg ($P<0.05$ and $P<0.01$, respectively), and the number of head-dips at a dose of 20 mg/kg ($P<0.05$). 2*S*-naringin and gossypin only presented a significant action at 30 mg/kg in reducing the number of rearings ($P<0.05$ and 0.01, respectively).

Spontaneous locomotor activity of mice was significantly reduced by 2*S*-hesperidin, at a dose of 5 mg/kg, ($P<0.01$). Diosmin, 2*S*-neohesperidin and rutin also reduced the ambulatory counts of mice, but with a higher dose compared with 2*S*-hesperidin (Table 2).

Considering the sedative effects obtained and the doses injected the activity of the glycosides assayed can be expressed by the following sequence, in decreasing order: 2*S*-hesperidin > linarin > rutin > diosmin ≅ 2*S*-neohesperidin > gossypin > 2*S*-naringin.

Notwithstanding the sedative action of the glycosides, the aglycones 2*S*-hesperetin, naringenin and quercetin, at a dose of 15 mg/kg, were unable to alter exploratory parameters in the hole board test (Fig. 2) or affect the locomotor activity assay (Table 2). Diosmetin, at the same dose, reduced significantly only the number of rearings ($P<0.01$, Fig. 2) and the locomotor counts ($P<0.01$, Table 2), although the number of head dips and time head dipping were apparently also decreased. Flavanone showed no effect on the spontaneous locomotor activity of mice at the dose tested (Table 2).

The dose of the aglycones tested (15 mg/kg) is equivalent in mass to the active dose of the corresponding glycoside (30 mg/kg).

3.3. *In vitro* interaction between flavonoids and $GABA_A$ receptors

In the [^{3}H]flunitrazepam binding assay the flavonoid glycosides: 2*S*-hesperidin, linarin, 2*S*-neohesperidin, 2*S*-naringin, rutin, diosmin and gossypin, and the aglycones: flavanone, 2*S*-hesperetin, acacetin, quercetin and diosmetin showed no potency in displacing the radioligand up to a concentration of 100 μM (data not shown).

In order to test their possible action as modulators of $GABA_A$ receptors, binding experiments with [^{3}H]TBOB, as well as electrophysiology studies, were performed.

The [^{3}H]TBOB binding assay is shown in Fig. 3. The IC_{50} values of GABA and diazepam, used as reference compounds, were 1.16 μM ($n=3$, 95% CI: 0.96–1.73 μM) and 278.8 μM ($n=3$, 95% CI: 137.1–566.7 μM) respectively. 2*S*-hesperidin, 2*S*-neohesperidin and diosmin were ineffective to displace [^{3}H]TBOB binding to well washed synaptosomal membranes from cerebral cortex with exogenous GABA added, up to a concentration of 1 mM ($n=3$), the same result was found in the absence of GABA (data not shown).

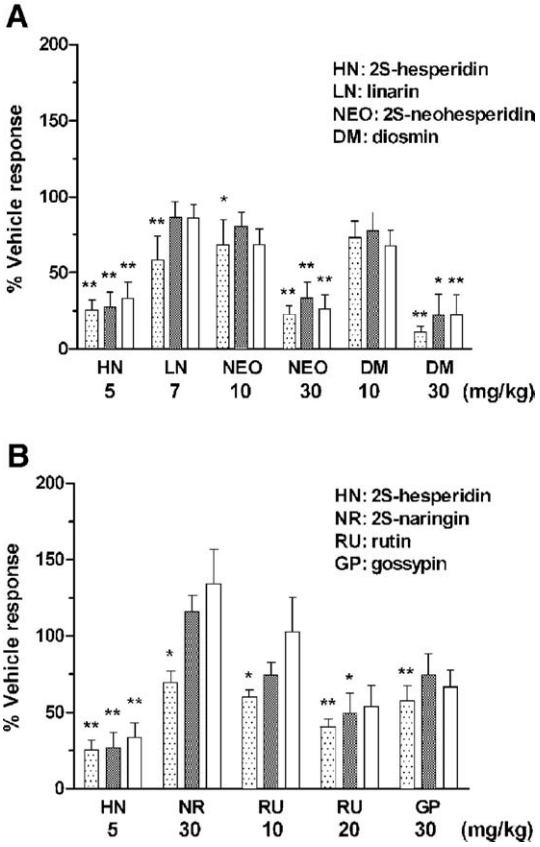


Fig. 1. Sedative effects in mice of flavonoid glycosides as measured in the hole board test. Results are expressed as percentages (mean±S.E.M.) of the vehicle response for the number of rearings (dotted bars), number of head-dips (grey bars) and time spent head-dipping (white bars), registered in a 5-min session. The individual doses of the compounds tested, administered i.p., are indicated in the abscissa. *$P<0.05$, **$P<0.01$, significantly different from vehicle; Newman-Keuls multiple comparison test after ANOVA. Number of animals per group ranged between 7 and 10, control animals were 30.

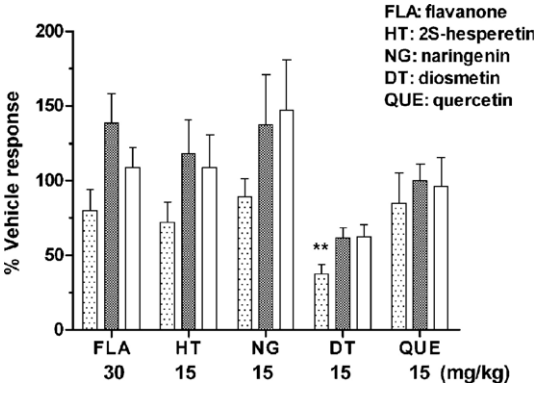


Fig. 2. Effect of aglycones in the hole board test. Results are expressed as percentages (mean±S.E.M.) of the vehicle response for the number of rearings (dotted bars), number of head-dips (grey bars) and time spent head-dipping (white bars) of mice, registered in a 5-min session in the hole board test performed 20 min after an i.p. injection of flavonoid aglycones. The individual doses of the compounds tested, administered i.p., are indicated in the abscissa. **$P<0.01$, significantly different from vehicle; Newman-Keuls multiple comparison test after ANOVA. Number of animals per group ranged between 7 and 10, control animals were 30.

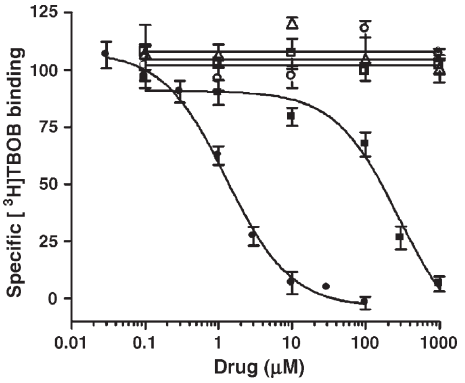


Fig. 3. Displacement curves generated for GABA (●), diazepam (■), 2*S*-hesperidin (○), 2*S*-neohesperidin (□) and diosmin (△) competition of [^{3}H]TBOB binding to well-washed rat cerebral cortex membranes with exogenous GABA added (0.3 μM). Results are expressed as percentages (mean±S.E.M.) of control specific [^{3}H]TBOB binding. GABA and diazepam were tested as reference compounds, and their IC_{50} values were 1.16 μM (95% CI: 0.96–1.73 μM) and 278.8 μM (95% CI: 137.1–566.7 μM), respectively.

Concerning the electrophysiological experiments, 2*S*-hesperidin, 2*S*-neohesperidin, diosmin and gossypin, at 300 μM, did not generate any response at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus* oocytes when administered alone (data not shown). To evaluate a possible modulatory action, the compounds were co-administered with a low concentration of GABA (6 μM). 2*S*-Hesperidin, 2*S*-neohesperidin and diosmin (at 100 and 300 μM) and gossypin (at 300 μM) did not modify the response generated by 6 μM GABA ($n=3$–6, $P>0.5$; Fig. 4).

3.4. Picrotoxin effect on the sedative action of hesperidin

Several doses of picrotoxin were tested to find the maximal dose devoid of intrinsic action. As shown in Table 3, picrotoxin at 0.7 mg/kg i.p. did not alter the performance of mice in the hole board and the locomotor activity tests.

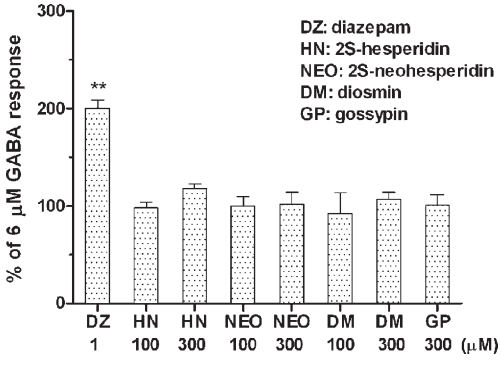


Fig. 4. Effect of diazepam, 2*S*-hesperidin, 2*S*-neohesperidin, diosmin and gossypin on GABA-elicited chloride currents at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus leavis* oocytes. Results are expressed as percentages (mean±S.E.M., $n=3$–6 oocytes) of the response generated by 6 μM GABA. Concentrations of the compounds tested are indicated in the abscissa. **$P<0.01$, significantly different from control; Newman-Keuls multiple comparison test after ANOVA.

Table 3
Picrotoxin effect on the sedative action of hesperidin

Pre-treatment/treatment	Hole board test (median±S.E.M.)			Locomotor activity test (median±S.E.M.)
	Number of rearings	Number of head-dips	Time head-dipping (s)	Counts
Vehicle/vehicle	33.7±0.3	15.7±1.4	14.1±3.6	718±26
Vehicle/2*S*-hesperidin	4.6±0.9[b]	4.0±1.9[a]	2.5±1.4[a]	354±96[a]
Picrotoxin/vehicle	33.3±4.7	16.3±2.2	11.2±0.7	662±15
Picrotoxin/2*S*-hesperidin	9.0±1.5[e]	3.8±1.6[d]	2.8±0.9[d]	386±51[c]

Performance of mice in the hole board and the locomotor activity tests after a pre-treatment with vehicle or picrotoxin (0.7 mg/kg, i.p.) and a treatment with vehicle or 2*S*-hesperidin (5 mg/kg, i.p.). Pre-treatment and treatment were administered in consecutive injections, 30 and 20 min, respectively, before testing.
[a]$P<0.01$, [b]$P<0.001$, significantly different from vehicle/vehicle; [c]$P<0.05$, [d]$P<0.01$, [e]$P<0.001$, significantly different from picrotoxin/vehicle; two-way ANOVA (pre-treatment vs. treatment) and post-hoc comparison was made using Bonferroni post-tests. Number of animals per group=8.

No significant interaction (pre-treatment vs. treatment) was found for all the parameters ($F_{1,13}=1.30$, $P>0.2$ for rearings; $F_{1,13}=0.04$, $P>0.5$ for head-dips; $F_{1,13}=0.93$, $P>0.3$ for time head-dipping and $F_{1,13}=1.30$, $P>0.2$ for locomotion). Only treatment (vehicle or 2*S*-hesperidin, 5 mg/kg, i.p.) was found to significantly change the response observed ($F_{1,13}=165.7$, $P<0.0001$ for rearings; $F_{1,13}=36.5$, $P<0.0001$ for head-dips; $F_{1,13}=35.5$, $P<0.0001$ for time head-dipping and $F_{1,13}=10.6$, $P<0.001$ for locomotor activity). Thus, pre-treatment (vehicle or picrotoxin) did not modify the response.

For those mice receiving vehicle in the pre-treatment there is a significant difference in the four parameters between those receiving vehicle or 2*S*-hesperidin in the treatment ($P<0.001$ for rearings, $P<0.01$ for head-dips, $P<0.01$ for time head-dipping and $P<0.01$ for locomotor activity; Table 3), hence the sedative action of 2*S*-hesperidin was observed.

For those mice receiving picrotoxin in the pre-treatment there is also a significant difference in the four parameters between those receiving vehicle or 2*S*-hesperidin in the treatment ($P<0.001$ for rearings, $P<0.01$ for head-dips, $P<0.01$ for time head-dipping and $P<0.05$ for locomotor activity). The values in Table 3 clearly show that picrotoxin does not antagonize 2*S*-hesperidin sedative action.

4. Discussion

The general aim of the studies reported here was to detect the possible CNS depressant action of a number of available flavonoid glycosides, compared with our previous reports on 2*S*-hesperidin and linarin (Marder et al., 2003; Fernández et al., 2004, 2005). We found that, 2*S*-neohesperidin, 2*S*-naringin, diosmin, rutin and gossypin, increased the thiopental-induced sleeping time in mice (Table 2), reduced the exploratory parameters in the hole board test (Fig. 1) and also reduced the spontaneous locomotor activity (Table 2). These results indicate that all the glycosides assayed cause a general inhibition of neuronal activity in the CNS.

Flavonoids are plant phytochemicals that cannot be synthesized by humans. Despite their widespread presence in our common diet, their sedative action is undetectable. This fact can be explained as follows:

– Dietary intake of flavonoids is estimated to reach 1–2 g/day (Havsteen, 2002) but the number of different compounds ingested varies, and all estimates currently available are incomplete (Peterson and Dwyer, 1998). The fact is that a normal diet, not including herbal teas, does not appear to provide sufficient amount of effective flavonoids for overt CNS effects.
– Metabolism of dietary flavonoids in humans is still obscure. It is assumed that glycosides are absorbed as their aglycones after prior hydrolysis of the sugar moiety along the digestive tract. Small amounts of the aglycones can also be present in the diet (Walle, 2004; Manach et al., 2005). The results presented here show that, except for diosmetin, the aglycones studied lack sedative action; hence their formation would decrease the central actions of the corresponding glycosides, clearly detectable when administered by the i.p. route.
– Hesperidin properties have been explored in many clinical and experimental conditions (Garg et al., 2001). Nevertheless, its remarkable activity on the CNS eluded detection. This failure may be partially explained by the fact that the drug generally used was the racemic variety provided by the citrus industry, and we found that the CNS active compound is the 2*S*-(−) isomer (Marder et al., 2003).

The $GABA_A$ receptor complex comprises a Cl^- channel and binding sites for several compounds, such as benzodiazepines, barbiturates, neuroactive steroids, and a variety of other drugs like loreclezole and propofol (Korpi et al., 2002). Furthermore, a site for convulsant drugs is also present in the complex. All these binding sites are allosterically coupled, resulting in a network of interactions that ultimately regulate the permeability of the Cl^- channel (Johnston, 2005).

Many flavone derivatives were found to be ligands for the $GABA_A$ receptors in the CNS; and to bind to the benzodiazepine binding site with resulting depressant actions in mice (Marder and Paladini, 2002; Johnston, 2005). In contrast, the active flavonoid glycosides studied in the present work were unable to modify [^{3}H]flunitrazepam binding to rat cerebral cortex synaptosomal membranes. According to our previous work on structure–activity relationships between flavonoids and their capacity to bind to the benzodiazepine binding site, this lack of affinity for the flumazenil-sensitive site can be explained by the presence of a sugar moiety of great molecular size; by the flavanone nucleus and a catechol group at C3′ and C4′ when present (Marder et al., 1996, 1997, 2001).

Electrophysiological experiments with flavone and flavanone derivatives have shown that some of them can modulate GABA-generated chloride currents, either positively or negatively (Goutman et al., 2003; Campbell et al., 2004; Kavvadias et al., 2004; Hall et al., 2005). Our functional studies performed with flavonoid glycosides show that they cannot directly activate $GABA_A$ receptors or modulate GABA-induced chloride currents in subtype $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *X. laevis* oocytes.

The functional state of a wide population of $GABA_A$ receptors subtypes, present in cortex membrane preparations, can be assayed by measuring the amount of [^{3}H]TBOB that binds to the convulsant site of this receptor (van Rijn and Willems-van Bree, 2003). The present results show that flavonoid glycosides do not modify [^{3}H]TBOB binding to rat cerebral synaptosomal membranes, associated with $GABA_A$ receptors.

Flavonoids glycosides are easily metabolized by the organism and it could be possible that secondary metabolites may activate $GABA_A$ receptors to mediate sedative effects. However, picrotoxin, a non-competitive antagonist, was unable to block the sedative actions of 2*S*-hesperidin in vivo at the doses and conditions used.

All the data discussed up to here strongly suggest that the CNS depressant action of flavonoid glycosides does not involve classical $GABA_A$ receptors, at least not directly.

Considering the sedative, the spontaneous locomotor activity and thiopental-induce sleeping time effects obtained with the flavonoid glycosides, the following decreasing order of action results:

2*S*-hesperidin > linarin > rutin > diosmin\cong 2*S*-neohesperidin > gossypin ≅ 2*S*-naringin.

Although there is not enough information to establish a full structure-sedative activity correlation, some partial conclusions are apparent from the behavioural results: linkage to a sugar moiety is important to preserve the action and according to the sequence obtained, the linkage between sugar residues is important. The most active glycosides had a 1→6 bond between rhamnose and glucose (Table 1). When this bond is changed to 1→2, like in 2*S*-neohesperidin, a remarkable decrease in activity is observed. Position of the sugar on the flavonoid nucleus seems relevant as well and position 7 is the most effective. Regarding the benzo-γ-pyrone portion of the molecules, the presence of a double bond between carbons 2 and 3, resulting in flavone derivatives with planar configuration (i.e. linarin) does not appear to be critical for activity. Hydroxylation and methoxylation of the benzo-γ-pyrone nucleus might be essential for activity; however the effects of changes in the substitution pattern could not be established here.

Flavonoid glycosides form the newest group within the growing family of flavonoids with activity on the CNS.

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