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Research report

Assessment of luteolin (3′,4′,5,7-tetrahydroxyflavone) neuropharmacological activity

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Abstract

Since the discovery that certain flavonoids (namely flavones) specifically recognise the central BDZ receptors, several efforts have been made to identify naturally occurring $GABA_A$ receptor benzodiazepine binding site ligands. Flavonoid derivatives with a flavone-like structure such as apigenin, chrysin and wogonin have been reported for their anxiolytic-like activity in different animal models of anxiety. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a widespread flavonoid aglycon that was reported as devoid of specific affinity for benzodiazepine receptor (BDZ-R) binding site, but its psychopharmacological activity is presently unknown. Considering (1) the close structural similarity with other active flavones, (2) the activity of some of its glycosilated derivatives and (3) the complexity of flavonoid effects in the central nervous system, luteolin was submitted to a battery of tests designed to evaluate its possible activity upon the CNS and its ability to interact with the BDZ-receptor binding sites was also analysed.

Luteolin apparently has CNS activity with anxiolytic-like effects despite the low affinity for the BDZ-R shown *in vitro*. Our findings suggest a possible interaction with other neurotransmitter systems but we cannot rule out the possibility that luteolin's metabolites might show a higher affinity for the BDZ-R *in vivo*, thus eliciting the evident anxiolytic-like effects through a GABAergic mechanism.

Keywords: Luteolin; Flavonoids; Flavones; Central nervous system; Anxiolytics; GABA

1. Introduction

Flavonoids are a large group of plant secondary metabolites that share a basic phenylbenzopyrone feature and are found in all vascular plants where they occur in several structurally and biosynthetically related classes [1]. They are important constituents of the human diet [2] and can also be found in expressive amounts in many medicinal plants [3]. Amongst the wide range of biological and pharmacological properties of these compounds we find a series of reports on their activity in the central nervous system (CNS) (for reviews see [4–6]). Since the discovery that certain flavonoids (namely flavones) specifically recognise the central BDZ receptors [7,8], efforts have been made to identify naturally occurring $GABA_A$ receptor benzodiazepine binding site ligands [5] to understand their interaction with these receptors [9–12] and to establish the CNS activity of different natural [13] and synthetic flavonoids [14,15]. Amongst these reports flavonoid derivatives with a flavone-like structure such as apigenin [16,17], chrysin [18] and wogonin [19] have been reported for their anxiolytic-like activity in different animal models of anxiety. These flavonoids with BDZ-receptor specificity and/or anxiolytic activity have been isolated from medicinal plants traditionally used in folk medicine for their anxiolytic/sedative properties such as *Passiflora coerulea* [20], *Matricaria recutita* [16], *Tilia tomentosa* [21], *Jatropha cilliata* [22], *Salvia guaranitica* [23], *Matricaria chamomilla* [17], *Zizyphus jujuba* [24]. Recently, we have reported on the isolation of luteolin-7-*O*-(2-rhamnosylglucoside) from *Passiflora edulis* Sims and demonstrated its anxiolytic-like activity [25].

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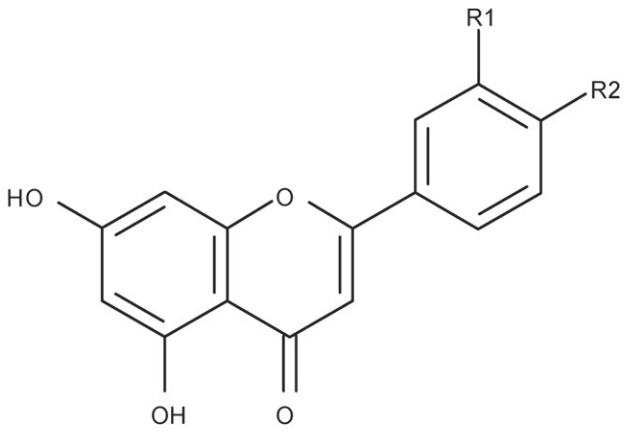

	R1	R2
Chrysin	-	-
Apigenin	-	OH
Luteolin	OH	OH

Fig. 1. Luteolin's structure is close to that of other flavones that have been reported for its anxyolitic activity like apigenin or chrysin.

Luteolin is a widespread flavonoid aglycon that was reported as devoid of specific affinity for BDZ-receptor binding site [21], but its psychopharmacological activity is presently unknown. Considering (1) the close structural similarity with other active flavones (Fig. 1), (2) the activity of some of its glycosilated derivatives [25,26,22] and (3) that flavonoid effects in the central nervous system are complex and can involve different mechanisms [27] besides the interaction with the benzodiazepine binding sites (BDZ-bs) at the $GABA_A$ receptors, we became interested in the possible psychopharmacological profile of action of luteolin. This substance, purchased from a commercial source, was submitted to a battery of tests designed to evaluate its possible activity upon the CNS and to an eventual understanding of mechanisms underlying its activity(ies). As we were also interested in analysing the ability of luteolin to interact with the BDZ-receptor binding sites, we have also evaluated this substance in a radioreceptor binding assay with [^{3}H]flunitrazepam.

2. Material and Methods

2.1. Animals

Male adult Swiss mice from our breeding stock, weighing 20–25 g, were used. Animals were placed in groups of 10 with free access to water and food, except during the experiments. They were kept on a 12/12 h day/night cycle (lights on at 07:00 a.m.) at controlled room temperature (23 ± 2 °C) and were allowed to adapt to the laboratory conditions for, at least, 1 week before the beginning of the behavioral experiments. Each animal was used just once. All experiments were conducted in accordance with international standards of animal welfare recommended by the Brazilian Society of Neuroscience and Behavior. The experimental protocols were approved by the local Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used and all behavioral testing was performed during the animal's day light period between 09:00 a.m. and 01:00 p.m.

2.2. Drugs

Diazepam i.v. solution (Dienpax ®, Sanofi-Winthrop Lab., Brazil) was diluted with distilled water and used in the dose of 1 mg/kg as reference drug (positive control) for anxiolytic, sedative, muscle relaxant and anticonvulsant activities. Luteolin, the flavonoid compound, was purchased from Extrasynthése (Genay, France). [^{3}H]flunitrazepam was obtained from Amersham Biosciences.

2.3. Treatments

Luteolin was freshly suspended (in an ultrasound bath) in a suitable amount of distilled water to be acutely (1 h) or repeatedly (14 days) administered *per os* (*p.o.*) by an intragastric cannula. Doses of luteolin (0.1–50 mg/kg) as well as the time intervals were determined in preliminary tests. Control groups received only distilled water in equivalent volumes by the same route. The behavioral tests were performed in a soundproof room between 09:00 a.m. and 01:00 p.m. to reduce the confounding influence of diurnal variation in spontaneous behavior.

2.4. Procedures

2.4.1. Motor performance evaluation

Muscle relaxant effects were evaluated using the horizontal-wire test that consists of a stretched copper wire placed 20 cm above the ground [28]. Motor coordination was assessed using a rota-rod apparatus. This equipment has a 2.5 cm bar, rotating at 12 rpm, divided in six parts and placed at a height of 25 cm. Latency to fall from the rotating bar and number of falls in a period of 1 min test were registered [29].

2.4.2. Elevated plus-maze test (EPM)

The elevated plus-maze was slightly modified from that used by Lister [30]. Briefly, it consisted of two open arms (30 cm × 5 cm × 0.25 cm) and two enclosed arms (30 cm × 5 cm × 15 cm), extending from a central platform (5 cm × 5 cm) and raised 50 cm above floor level. The maze floor was constructed from black Plexiglas and the walls from clear Plexiglas. The conventional spatial–temporal measures recorded were the number of entries (all four paws on open or enclosed arms and expressed as percentage of total entries), the time spent on open arms (expressed as percentage of time spent on closed plus open arms), number of entries on enclosed arms and the time on the central platform. Ethologically derived measures were grooming, rearing, stretched attend postures (SAP), head-dipping (HD) and defecation as an emotionally related parameter [31]. A selective increase in the parameters of exploration of the open arms of the maze reveals an anxiolytic effect [32].

2.4.3. Hole-board test

The hole-board consisted of a square box made of transparent Plexiglas (50 cm × 50 cm × 30 cm), 10 cm above table surface, with equally distributed nine holes, 2 cm in diameter. The area of the hole-board is divided with white ink into 24 smaller areas. During 5 min we registered the number of head-dips, grooming behavior, rears and also of displacements between the different areas (locomotor activity) [33].

2.4.4. Potentiation of barbiturate-induced loss of righting reflex

One hour after treatment with luteolin, animals were administered (i.p.) with sodium pentobarbital (50 mg/kg). Latency for the loss of the righting reflex and its total duration was registered for three consecutive hours [34].

2.4.5. Catalepsy test

Animals' forepaws were placed over a horizontal glass tube standing 5 cm above floor surface, each 10 min interval for up 1 h. Catalepsy was evaluated as the time until removal of the forefeet from the tube. Two different sets of

experiments were performed: (1) animals were treated with 5, 10 and 50 mg/kg of pure luteolin and submitted to the test conditions; (2) animals previously treated with luteolin were administered with haloperidol (1 mg/kg) immediately before testing [35].

2.4.6. Maximal electroshock test

One hour after treatment, mice were submitted to a transcorneal electrical stimulation (50 mA; 0.2 s: 60 Hz). The flexion time (flexion of the front limbs) and the extension time (full hind limbs extension) of the convulsions elicited by the electrical stimulus, as well as the incidence and lethality of the induced convulsions, were registered [36].

2.4.7. Forced-swimming test

The test was slightly modified from that proposed by Porsolt et al. [37] and consisted in one exposure (6 min) to a water tank (height, 35 cm; diameter, 24 cm, with a water column of 13.5 cm at 25 °C). We have registered total immobilization time and the latency for this behavior after the first minute.

2.4.8. Rectal temperature evaluation

Body temperature was measured through a glycerin-lubricated thermistor (Lumiscope 2018) probe inserted about 1 cm into the rectum of the animal immediately before (basal values) and 1 h after treatment.

2.4.9. Statistical analysis

Data were analysed with Graphpad Prism® (v4.03). The statistical tests used were one-way ANOVA followed by Dunnett's test for comparison of treatment groups with control and Tukey's test for comparison between all treatment groups. Radioligand binding data were analysed by non-linear regression tools provided by the same software.

2.4.10. In vitro radioreceptor binding assay

Crude synaptic membranes were prepared from isolated rat brain cortices as previously described elsewhere [38].

Binding assays were performed using a semi-automatic filtration technique with diazepam (100 μM) to obtain the specific binding. Competition curves were obtained by adding to the assay tubes buffer solution (40 mM Hepes-Tris, pH 7.4), luteolin (5.55 μM–3.5 mM) or diazepam, followed by [^{3}H]flunitrazepam (88 Ci/mmol—final concentration 1.5 nM in the inhibition curves and 0.014–20 nM in the saturation curves) and finally brain tissue homogenate (about 300–400 mg protein) was added to initiate binding. The assays were done in triplicate and tubes were incubated at 37 °C during 30 min and terminated by rapid filtration through glass fiber filters. The radioactivity remaining in the filters was determined in 8 ml of scintillation liquid (toluene 1 L, 167 mg of 2,5-difeniloxazol 7.3 g, *p*-bis(2(5-feniloxazoil(-benzene and 250 ml of Triton X-100) in a Packard Tri-Carb 2500 TR scintillation counter.

3. Results

3.1. Motor performance evaluation

Acute treatment with luteolin (1–50 mg/kg) did not affect the motor coordination or muscle relaxation of the animals, as measured on the rota-rod (ANOVA: $F_{4.38} = 1.705$; $p > 0.05$) and horizontal-wire tests (ANOVA: $F_{4.38} = 0.7528$; $p > 0.05$).

Also, there were no changes in the parameters directly related with motor activity in the hole-board test (number of crossings between the different sections) (ANOVA: $F_{4.38} = 0.8242$; $p > 0.05$) and in the elevated plus-maze (total number of entries in the closed arms of the maze: data not shown; ANOVA: $F_{4.38} = 1.311$; $p > 0.05$).

3.2. Anxiolytic activity

ANOVA showed a significant difference within treated groups ($F_{5.46} = 6.705$; $p < 0.0001$) and Dunnett's test revealed that only with the dose of 5 mg/kg there was a significant increase ($p < 0.05$) in the percentage of entries in the open areas of the EPM after acute treatment with luteolin (Fig. 2). As for the percentage of time spent in those areas we could not find significant differences between luteolin-treated groups and control groups ($F_{4.38} = 1.624$; $p > 0.05$). ANOVA analysis of ethological parameters namely unprotected head-dipping and stretch approach postures also revealed significant differences when compared with the control group ($F_{4.38} = 4.264$ and 3.486, with $p = 0.0060$ and 0.0021, respectively) and Dunnett's test showed there was a significant increase in unprotected head-dipping ($p < 0.05$) and a decrease in the stretch approach postures ($p < 0.05$) displayed after the administration of this same dose (Table 1).

3.3. Sedative activity

ANOVA showed a significant difference within treated groups ($F_{6.50} = 13.83$; $p < 0.0001$) and with Tukey's multiple comparison test we could observe a significant reduction of the latency time for the pentobarbital-induced loss of righting reflex, even with relatively low doses of luteolin (0.1 mg/kg; $p < 0.05$) and a more pronounced effect when doses higher

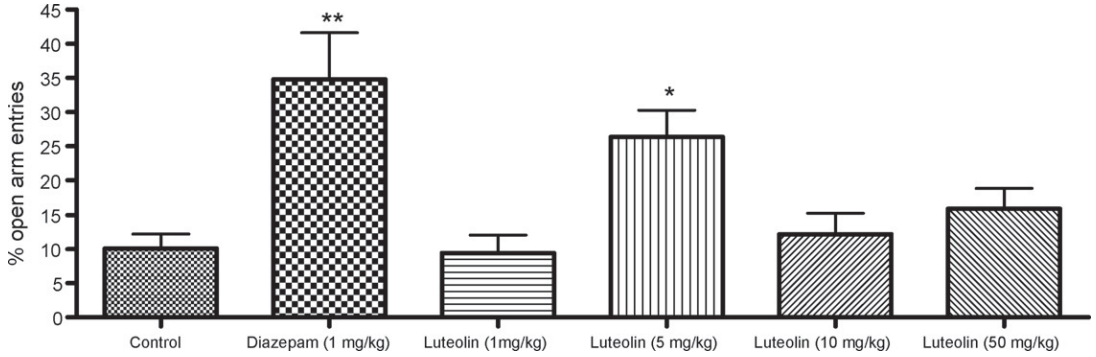

Fig. 2. Performance of mice in the elevated plus-maze after acute treatment with 1, 5, 10 and 50 mg/kg of luteolin. The percentage of entries in the open area was calculated with relation to the total number of entries in both closed and open arms of the maze. Results are expressed as mean ± S.E.M. ($n = 8$–10). *$p < 0.05$; **$p < 0.01$ versus control (vehicle treated group; Dunnett's test).

Table 1
Ethologically derived measures in the elevated plus-maze

	Control	Luteolin 1 mg/kg	Luteolin 5 mg/kg	Luteolin 10 mg/kg	Luteolin 50 mg/kg
Protected head-dipping	12.6 ± 3.7	13.98 ± 4.9	9.3 ± 2.9	9.7 ± 3.9	11.3 ± 4.8
Unprotected head-dipping	0.8 ± 0.7	0.4 ± 0.5	2.9 ± 0.6*	1.6 ± 1.5	0.8 ± 0.6
Rearing	22.8 ± 7.0	19.4 ± 5.5	27.7 ± 5.8	18.0 ± 4.0	20.8 ± 4.2
Immobility	0.6 ± 1.0	1.4 ± 1.8	1,7 ± 2.2	2.5 ± 1.5	6.0 ± 6.4
Grooming	4.2 ± 4.2	4.0 ± 3.7	0.7 ± 0.9	1.3 ± 1.3	4.6 ± 4.2
SAP	9.4 ± 2.5	5.5 ± 4.7	4.3 ± 1.1*	11.2 ± 3.4	12.3 ± 4.1

Results are expressed as mean ± S.E.M. ($n = 8$–10). *$p < 0.05$ versus control (vehicle treated group) (Dunnett's test).

than 1 mg/kg were tested (Fig. 3), as concluded from the fact that the effect observed after the administration of luteolin 0.1 mg/kg was significantly lower than after luteolin 5 and 10 mg/kg ($p < 0.05$). ANOVA analysis of the total duration of the pentobarbital-induced loss of righting reflex data showed significant differences between treatment groups ($F_{6.50} = 17.31$; $p < 0.0001$); however, the total duration was only significantly increased with high doses of luteolin (10 mg/kg) ($p < 0.05$, with Dunnett's test) (Fig. 4). Also, we have observed that during the loss of righting reflex all the animals treated with luteolin and, particularly, groups that received the dose of 5 and 10 mg/kg, showed unusual tremors of both anterior and posterior limbs.

3.4. Anticonvulsant activity

Compared with control groups, animals treated with luteolin showed no differences in the flexion and extension times as well as in the lethality of the electroshock-induced seizures for any of the doses tested (data not shown).

3.5. Catalepsy test

Luteolin (5, 10 and 50 mg/kg) did not induce catalepsy *per se*, but analysing the results with ANOVA and Dunnett's test we could conclude that the dose of 5 mg/kg significantly antagonised catalepsy induced by haloperidol ($F_{3.24} = 8.201$; $p < 0.01$)

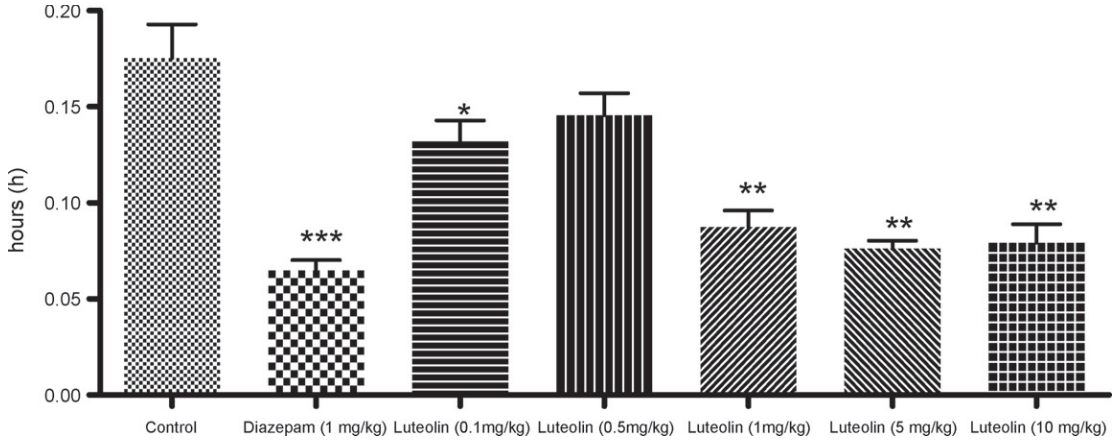

Fig. 3. Latency to the loss of the righting reflex induced by pentobarbital. Results are expressed as mean ± S.E.M. ($n = 7$–11). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control (vehicle treated group; Tukey's test).

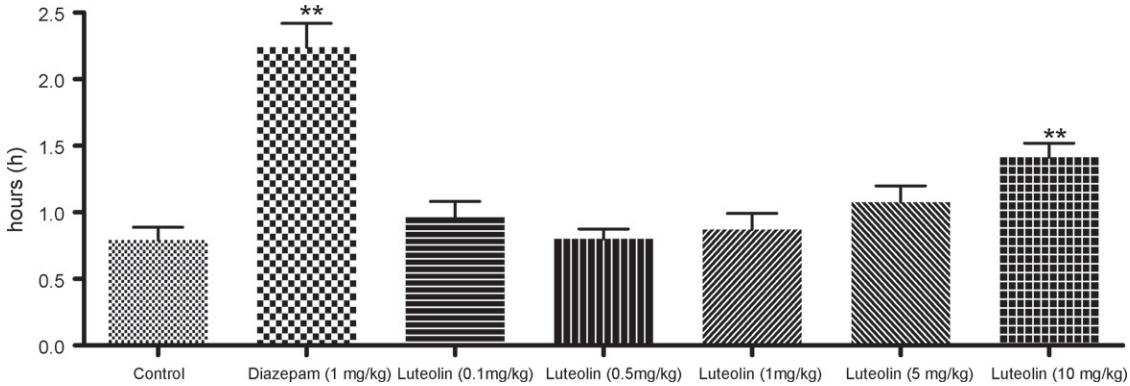

Fig. 4. Total duration of the loss of the righting reflex induced by pentobarbital. Results are expressed as mean ± S.E.M. ($n = 7$–11). **$p < 0.01$ versus control (vehicle treated group; Dunnett's test).

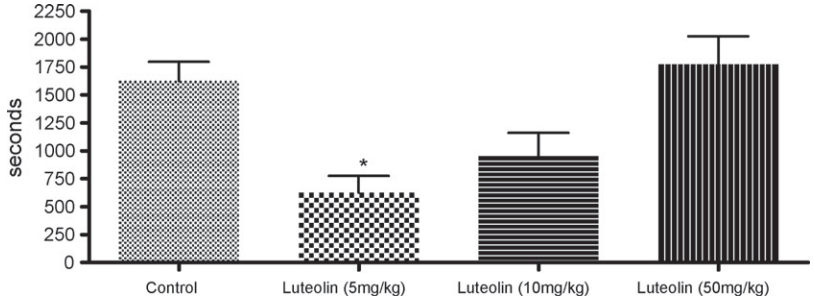

Fig. 5. Antagonism of haloperidol-induced catalepsy. Catalepsy was evaluated as the time of involuntary permanence of the animal in an unusual position (time until removal of the forefeet of the tube). Results are expressed as mean ± S.E.M. ($n = 6$–8). *$p < 0.01$ versus control (vehicle treated group; Dunnett's test).

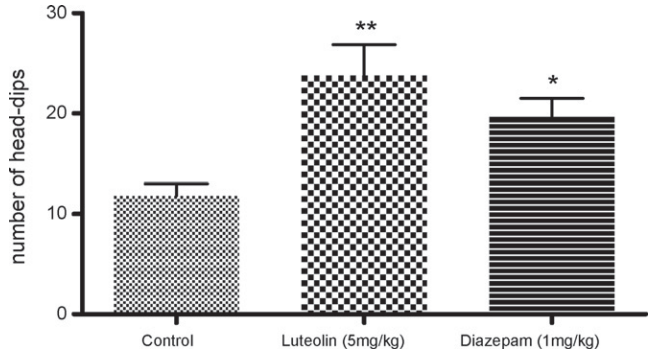

Fig. 6. Effect of chronic treatment with luteolin (5 mg/kg) on the frequency of head-dips in the hole-board test. Results are expressed as mean ± S.E.M. ($n = 9$–11). *$p < 0.05$; **$p < 0.01$ versus control (vehicle treated group; Dunnett's test).

and this effect disappeared when higher doses (10, 50 mg/kg) ($F_{3.24} = 8.201$; $p > 0.05$) were tested (Fig. 5).

3.6. Chronic treatment

Animals treated with luteolin (5 mg/kg) daily for 14 days did not exhibit any significant changes either on the motor activity parameters as evaluated in the rota-rod ($F_{2.27} = 0.967$; $p > 0.05$) and horizontal-wire ($F_{2.27} = 0.822$; $p > 0.05$) tests or on the parameters related with motor activity in the EPM and hole-board test (data not shown).

Also, both ethological and spatio-temporal parameters of the EPM related with anxiolytic-like effects remained unchanged after chronic treatment with luteolin (5 mg/kg); however, in the hole-board test, analysis of the results with ANOVA and Dunnett's test showed a significant increase in head-dipping ($F_{2.27} = 7.987$; $p < 0.01$; Fig. 6).

On the contrary, in the forced-swimming test, the ANOVA showed significant differences within treatment groups for both parameters measured ($F_{2.27} = 10.26$; $p = 0.0005$ and $F_{2.27} = 4.236$; $p = 0.0292$, for total immobilization time and latency to immobilization, respectively) and Dunnett's test revealed that repeated treatment with luteolin (5 mg/kg) significantly reduced the latency to immobilization ($p < 0.05$) and increased the total time of immobilization ($p < 0.01$; Fig. 7).

3.7. Radioreceptor binding assay

From [^{3}H]flunitrazepam saturation binding experiments K_d and B_{max} determined were 13.9 ± 3.3 nM and 10316 ± 1186 cpm, respectively. In competitive binding experiments carried out in the presence of 5 nM of [^{3}H]flunitrazepam, luteolin inhibited this radioligand binding to the rat cerebral cortex membranes (Fig. 8) with a K_i of 60.1 μM and a Hill slope of −0.91.

4. Discussion

The results in the different tests show that luteolin after both acute and chronic treatment is devoid of muscle relaxant or motor

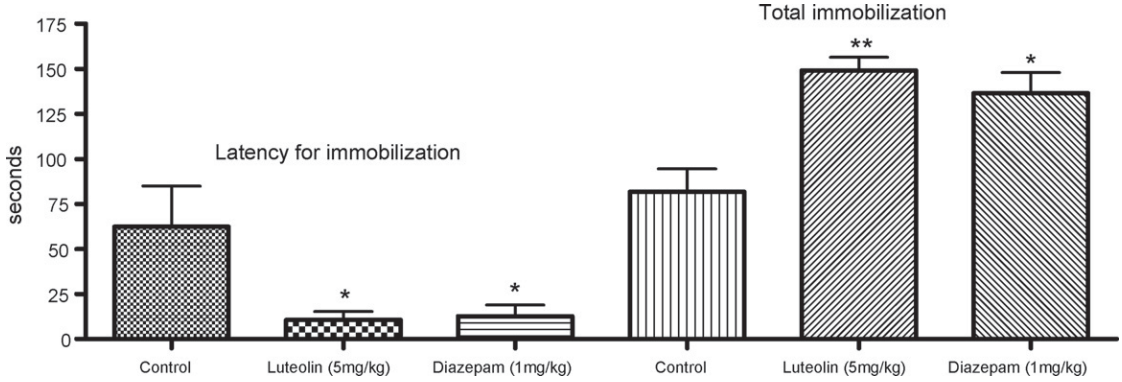

Fig. 7. Effect of chronic treatment with luteolin in the performance of mice in the forced-swimming test. We have registered total immobilization time and the latency to this behavior after the first minute. A mouse was considered to be immobile when it floated or made only small movements necessary to keep its head above water. Results are expressed as mean ± S.E.M. ($n = 9$–11). *$p < 0.05$; **$p < 0.01$ versus control (vehicle treated group; Dunnett's test).

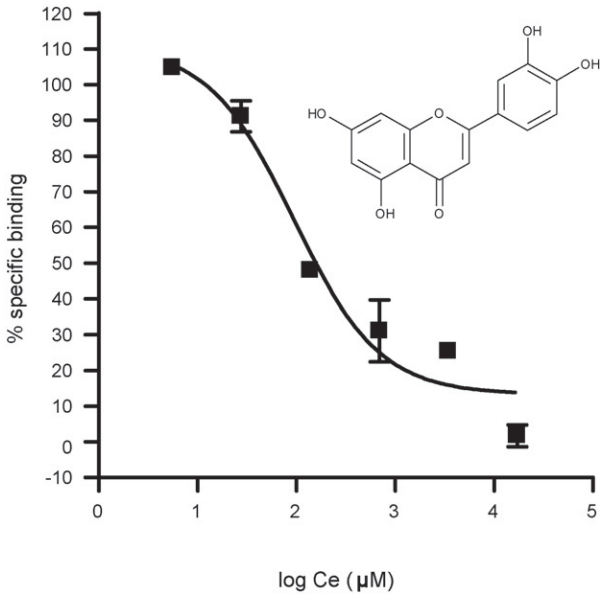

Fig. 8. Structure of luteolin and competitive inhibition curve of [^{3}H]flunitrazepam binding to synaptosomal membranes.

coordination effects and activity on the CNS is thus not hindering motor activity performance.

Increased exploration in the open areas of the EPM (% open arm entries), diminution of SAP and increase in unprotected head-dipping are all consistent and suggest an anxiolytic-like effect [39]. However, one should expect the increased number of entries in the open areas of the maze also to reflect significantly in the percentage of time spent there; but that was not the case and, also, we could not observe any clear dose–effect relation in this test.

Potentiation of pentobarbital-induced loss of righting reflex can be elicited through interaction with different neurotransmitter systems, namely GABA [40,41] or 5-HT [42]. In our tests, the reduced latency time for the pentobarbital-induced loss of righting reflex elicited by luteolin (0.1, 5 and 10 mg/kg) and the increase in the total duration of this effect (10 mg/kg) can be interpreted as an indication of luteolin's possible interference with these systems.

Unlike classical benzodiazepines [43] and other flavone type BDZ-R ligands like apigenin [17] or chrysin [18], luteolin failed to give any protection against maximal electroshock.

Haloperidol is a potent D_2 antagonist [44] that elicits catalepsy. Besides dopamine, several neurotransmitters like serotonin, acetylcholine, GABA or endorphins are found to be involved in the expression of catalepsy and haloperidol-induced catalepsy can be blocked by such diverse drugs as selective dopamine D_3 receptor antagonists [45], 5-HT_{1A} agonists [46,47], anti-cholinergics [48] or A_{2A} receptor antagonists [49]. There are also well-known interactions between the GABAergic and dopaminergic system [50,51] and classic benzodiazepines like diazepam potentiate haloperidol-induced catalepsy [52]. On the contrary, $GABA_A$ agonists like muscimol are reported to antagonise haloperidol-induced catalepsy at low doses with reverse effects at higher doses [53] and, interestingly enough, $GABA_B$ agonist baclofen antagonises the action of haloperidol in a low dose (1 mg/kg) without any visible effect in higher doses (2–8 mg/kg) [54]. There are reports of other flavonoid-type molecules like quercetin [55] or flavonoid-enriched extracts [56] interfering with haloperidol-induced catalepsy with different outcomes but the present results suggest that luteolin has a baclofen-like effect in this test. However, it must be noted that unlike baclofen [57], luteolin did not produce any significant reduction in the animals' body temperature (results not shown).

After the chronic treatment, our results of the hole-board test suggest an anxiolytic-like effect [58] but these were not observed in the EPM. Normally, the results with anxiolytics in both tests seem to correlate well but their sensitivity can differ [59] and that could explain these somewhat surprising results. On the contrary, it was previously reported that handling history can modify the behavioral effects of drugs in the EPM and $GABA_B$ agonists like baclofen apparently exert an anxiolytic-like effect in this test only in handling naïve rats [60] and this could also explain the different results in the EPM after acute or repeated treatment. Moreover, the development of tolerance to the repeated treatment with luteolin could also explain its lack of effect in the EPM since tolerance is observed with the benzodiazepine drugs [61].

The meaning of immobility in swimming tests may vary in accordance with the protocol reflecting helplessness or adaptation in the forced-swimming test or in the swimming stress, respectively [62]. For mice, the forced-swimming conditions used in this test resemble more closely the situation of swimming-stress (once the animal does not touches the bottom with its hind paws) and in these conditions drugs like diazepam have the ability to increase immobility time [62,63] just as it was observed in our assay. Other structurally related compounds like apigenin, upon acute treatment with relatively high doses (25 mg/kg), have induced an antidepressant-like activity (reduction in immobility time) in the forced-swimming test [64].

4.1. Radioreceptor binding assay

Luteolin had previously been reported not to displace [^{3}H]flunitrazepam binding to central benzodiazepine receptors (BDZ-R) ($IC_{50} > 100\ \mu M$) [21]. In our experiments we have determined that luteolin has in fact the ability to displace [^{3}H]flunitrazepam binding, though exhibiting a low affinity for these receptors, with a K_i in the high (60.1) μM range. Despite the need to further analyse luteolin's interaction with BDZ-R, our results suggest that by itself this interaction does not seem to fully explain the results observed *in vivo*, thus prompting renewed interest in the analysis of possible interactions with other receptors.

Most of the literature published concerning the anxiolytic-like activity of flavone-type compounds has focused on the ability of these molecules to interact with the $GABA_A$ benzodiazepine binding site (BDZ-bs) (for review see [5]). However, Luteolin, despite its low affinity for the BDZ-R, seems to have anxiolytic-like effects or, at least, to interact with different neurotransmitter systems so as to induce CNS effects. Another neurotransmitter system such as the 5-HT receptors [65,66]

could be involved in its action, and this should be further investigated. On the contrary, we must consider that flavonoids are subject to intense metabolism [67] and after oral administration of luteolin to rats, free luteolin has been determined in plasma but also luteolin's sulfate and glucoronate derivatives (the main metabolite was found to be a luteolin monoglucoronate) as well as *o*-methyl luteolin, with the dose administered strongly affecting the type of metabolites formed [68]. As there is no information about the affinity for the BDZ-receptor of luteolin's metabolites we cannot at this point discard the hypothesis that these might exhibit higher affinities for the BDZ-receptor, thus eliciting the evidenced anxiolytic-like effects through a GABAergic mechanism and this aspect should also be further investigated.

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