The Anti-Immobility Effect of Hyperoside on the Forced Swimming Test in Rats is Mediated by the D2-Like Receptors Activation

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Key words
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- Guttiferae
- *Hypericum caprifoliatum*
- hyperoside
- antidepressant-like activity

Abstract

The crude extracts of *Hypericum* species native to South Brazil showed analgesic and antidepressant-like effects in rodents. The chemical characterization of these species revealed that they are rich in flavonoids and phloroglucinol derivatives. In the present study a detailed investigation was performed on the activities of hyperoside (HYP), a common flavonoid in the genus *Hypericum*. Hyperoside was obtained from the aerial parts of *H. caprifoliatum* by chromatographic procedures. Mice treated with single doses (10, 20 and 40 mg/kg i.p.) did not present signs of toxicity or weight loss. At 20 and 40 mg/kg i.p. the mice exploratory behavior in the open field test was reduced. At 20 mg/kg i. p. the pentobarbital sleeping time increased, but not the sleeping latency. No activity was found on the hot-plate (10 and 20 mg/kg i.p.) or in the acetic acid-induced writhing test (20 and 40 mg/kg p.o.). Nevertheless, an antidepressant-like effect in the forced swimming test in mice and rats was observed (HYP 10 and 20 mg/kg i.p. in mice; HYP 1.8 mg/kg/day p.o. in rats). The antidepressant-like effect in rats was prevented by the administration of sulpiride (50 mg/kg i.p.) a D2 antagonist. In conclusion, hyperoside was found to present a depressor effect on the central nervous system as well as an antidepressant-like effect in rodents which is, at least in part, mediated by the dopaminergic system.

Abbreviations

ACTH:	adrenocorticotropic hormone
DBH:	dopamine-beta-hydroxylase
FST:	forced swimming test
HP1:	6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran
HPA:	hypothalamic-pituitary-adrenal
HYP:	hyperoside
SULP:	sulpiride

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Introduction

Hyperoside (quercetin 3-*O*-galactoside) (HYP) is one of the major components of *Hypericum perforatum* (St. John's wort) and also of South Brazilian *Hypericum* species (family Guttiferae) [1]. The efficacy of *H. perforatum* as an antidepressant has been demonstrated in numerous clinical studies and meta-analyses [2,3]. Nowadays, the pharmacological actions of this species are attributed to a variety of constituents rather than to a single compound. The phloroglucinol derivatives hyperforin and adhyperforin, the naphthodianthrones hypericin and pseudohypericin, the flavonoids hyperoside, rutin, quercitrin and isoquercitrin have all been considered as active ingredients of the plant [4]. Some data strongly indicate hyperforin and derivatives as the most active compounds [5] while other studies pointed at the flavonoids, such as hyperoside and rutin, as being responsible for the activity [6].

In South Brazil, 20 *Hypericum* species have been identified. Although in popular medicine the use of these species for antidepressant purposes has not been documented, previous experiments carried out in our laboratory demonstrated that the methanol [7,8] and cyclohexane extracts of the native *Hypericum* species presented antidepressant-like action in rodents via dopaminergic neurotransmission [9]. *Hypericum polyanthemum* and *H. caprifoliatum* extracts also displayed antinociceptive effects. The effect of the methanolic extract from *H. caprifoliatum* was only partially prevented by naloxone suggesting that this extract contains at least two groups of substances acting by different mechanisms [10]. The chemi-

cal characterization revealed that the above-mentioned species are rich in phloroglucinol derivatives, benzopyrans and flavonoids, with hyperoside being the major component of the methanol extracts [1,11]. HP1 (6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran), the main benzopyran isolated from the cyclohexane extract of *H. polyanthemum* showed an antinociceptive effect in the hot-plate and writhing tests in mice, which was counteracted by naloxone, thus indicating that it acts through the opioid system [12].

Rylski and coworkers [13] demonstrated the activity of hyperoside in the hot-plate test at doses ranging from 3.5–10 mg/kg i.p. Several studies attributed anti-inflammatory properties to hyperoside. It presented a low topical anti-inflammatory activity in croton oil-induced mouse ear edema [14], reduced prostaglandin E2 (PGE2) production [15], inhibited the increase of acetic acid-induced vascular permeability in mice [16], and also suppressed LPS-induced nitrite production [17]. A mixture of hyperoside and isoquercitrin inhibited *p*-benzoquinone-induced writhings in mice in 25.8% as well as reduced carrageenan-induced paw edema in 26.8–29.7%, and also inhibited 12-*O*-tetradecanoyl 13-acetate (TPA)-induced mouse ear edema in 32.8% [18].

In this study we have carried out a reassessment of the antinociceptive and antidepressant-like effects of hyperoside by testing doses and animal species different from those evaluated by others [6,13]. Hyperoside was evaluated in the hot-plate and acetic acid-induced writhing tests in mice, as well as in the forced swimming test (FST) in mice and rats. The involvement of D2-like dopamine receptors on the antidepressant-like effect in the forced swimming test was also investigated.

Materials and Methods

Plant material

Aerial parts of *H. caprifoliatum* Cham. & Schlecht. were collected in November 2007, in the region of Viamão, South Brazil. The plant material was identified by Dr. Sérgio Bordignon (ULBRA-RS-BRASIL) and voucher specimens were deposited in the herbarium of the Federal University of Rio Grande do Sul (ICN) (Bordignon 1496). The plant collection was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (N° 03/2008).

Extraction and isolation

The dried and powdered plant material (300 g of aerial parts) was successively extracted by maceration with dichloromethane (1 : 10 w/v; 3 × 24 h) to remove the lipophilic compounds, and then with methanol (1 : 10 w/v; 3 × 24 h) at room temperature (25 °C). The methanol extract, concentrated under reduced pressure, afforded a fraction rich in flavonoids. Hyperoside was purified by precipitation and subsequent silica gel column chromatography GF_{60} (Merck®) (50 × 3 cm) by using an ethyl acetate-methanol gradient system as the mobile phase. Hyperoside (1200 mg: 0.4 g/100 g raw material) was identified by TLC, ^{1}H-NMR and ^{13}C-NMR.

NMR data (400 MHz for ^{1}H and 100 MHz for ^{13}C) were measured on a JEOL Eclipse 400 spectrometer in MeOD using the solvent peaks as the internal standard. TLC: silica gel 60 F_{254} precoated, ethyl acetate-acetic acid-formic acid-water, 100 : 11 : 11 : 26 v/v/v/v. Bands were detected under UV light (254 nm).

Behavioral experiments

Animals: Adult male CF1 mice (25–30 g) and male Wistar rats (weight 200–300 g) purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS – RS, Brazil) colony were used. The animals were housed in plastic cages in groups of eight mice (17 × 28 × 13 cm) or five rats (42 × 28 × 16 cm), under a 12-h light/dark cycle (lights on at 7 : 00 a.m.), at 22 ± 1 °C, with free access to standard certified rodent diet (Nuvital®) and tap water. All behavioral experiments were performed according to the guidelines of The National Research Ethical Committee (published by National Heath Council – MS, 1998) and Brazilian Law [19], which are in compliance with the International Guiding Principles for Biomedical Research Involving Animals [20]. All protocols were approved by the UFRGS Ethical Committee (N° 2008008).

Drugs and treatments: The following drugs were used: pentobarbital (Cristália), diazepam (Cristália), morphine (Cristália), dipyrone (Sanofi-Aventis), imipramine (Galena). Hyperoside was dissolved in saline solution containing 1% polysorbate 80. The pH of the final solution was 5.0. The negative control group received vehicle i.p. or p.o. (saline containing 1% polysorbate 80). The other drugs were dissolved in saline (NaCl 0.9%) solution immediately before use. All administered drugs presented a pharmaceutical grade of purity (> 97%). The volume administered was 10 mL/kg for mice and 1 mL/kg for rats according to Viana and coworkers [9]. Hyperoside doses were chosen based on Butterweck and coworkers [6], Rylski and coworkers [13] and Chang and coworkers [21].

Gross behavior observation: Groups of mice were treated with a single dose of hyperoside 10, 20 or 40 mg/kg i.p. or vehicle i.p. and observed for 2 h with no interruption. After that, animals were observed 6 and 12 h after treatment and everyday for 14 days. Death occurrence and toxicity signs such as piloerection, palpebral ptoses, abdominal contortions, locomotion, hypothermia, muscular tonus, shacking, posterior paws paralyzation, salivation, bronchial secretion and convulsions were considered. The body weight was also registered.

Locomotor activity: The Swiss CF1 mice were exposed to the open field immediately after the treatments (HYP 10, 20 or 40 mg/kg i.p., or vehicle i.p.). The animals were left to explore the open field freely for 20 min. The numbers of line crossings, rearings and groomings were counted. The first 5 min were not considered (habituation period).

Pentobarbital sleeping time: Groups of male Swiss mice were treated with HYP 10, 20 or 40 mg/kg i.p., or vehicle i.p. Thirty minutes after the treatment all groups received pentobarbital (40 mg/kg i.p.) and the time elapsed between the loss and voluntary recovery of the righting reflex was recorded as sleeping time. A ceiling of 240 min was set. The sleep latency was also recorded.

Hot-plate test: Firstly, the mice were habituated to the nonfunctioning hot plate apparatus for 1 min. Thirty minutes later the animals were placed on the functioning hot plate (Ugo Basile) (55 ± 1 °C) to determine baseline responsiveness. The time elapsed until the animal licked one of its hind paws or jumped was recorded (latency time, in seconds = basal latency). Mice that presented a baseline reaction of more than 20 s were eliminated. Subsequently, the animals received HYP 10 or 20 mg/kg i.p., vehicle i.p. or morphine 4 mg/kg i.p. Thirty minutes later they were placed on the hot plate. A maximum latency time of 40 s was imposed.

Writhing test: The animals were treated with HYP 20 or 40 mg/kg p.o., dipyrone 150 mg/kg p.o. or vehicle p.o. 45 min before receiv-

ing an intraperitoneal acetic acid injection (0.8%, 10 mL/kg) (Merck AG). Mice were then individually placed in glass observation chambers and observed during 15 min in which the number of abdominal writhes was counted. The percentage analgesic activity was calculated as follows:

$$\text{Percentage analgesic activity} = \left(\frac{N - N' \times 100}{N}\right)$$

where N represents the average number of writhings of the control group and N' the average number of stretchings of the test group.

Forced swimming test: Rats and mice were submitted to forced swimming according to Porsolt's procedure [22,23] with minor modifications.

In rats it was performed as described by Daudt and coworkers [7]. The rats were submitted to swimming for 15 min in water with a temperature of 22 ± 1 °C and depth of 30 cm. The treatment was administered 5 min, 19 and 23 h after the first swimming exposition. One hour after the last injection (24 h after the first swimming session), the animals were submitted to a second swimming exposition (5 min), and their immobility time was measured.

Different groups were treated with HYP 1.8 mg/kg/day, p.o. (three adminstrations of 0.6 mg/kg) according to Butterweck and coworkers [6], imipramine 60 mg/kg/day p.o. (three administrations of 20 mg/kg) or vehicle p.o. To assess the involvement of the dopaminergic neurotransmission, rats were treated with sulpiride (50 mg/kg, i.p.) [9] 30 min before receiving the last treatment.

In mice the animals were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 22 ± 1 °C; the immobility duration was recorded during a 6-min period. Mice were treated with HYP (10 or 20 mg/kg i.p.), imipramine (20 mg/kg i.p.), or vehicle i.p. 30 min before swimming.

Statistical analysis

The statistics were performed according to the data distribution. The data from pentobarbital sleeping time and FST in rats (asymptotically F distributed) were analyzed by one-way Welch's ANOVA followed by a Dunnet post hoc test. The results from writhing and FST in mice were analyzed by one-way ANOVA followed by Student-Newman-Keuls post hoc test, while statistical comparison of hot-plate data was performed by two-way repeated measures ANOVA followed by Student-Newman-Keuls post hoc test. The analyses were performed using Sigma Stat 2.03 software (Jandel Scientific Corporation). Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

In this study we have validated previous rather qualitative reports by Dall'Agnol and coworkers [1] that reported hyperoside (**Fig. 1**) as one of the main compounds from *H. caprifoliatum* methanol extract. It constitutes 0.4% of the dried aerial parts. The identity and purity of the chemical structure was confirmed by ^{1}H- and ^{13}C-NMR experiments and by comparing these spectroscopic data with those cited in the literature [24].

We have evaluated this flavonoid in several behavioral experiments in rats and mice at different routes and doses since pharmacokinetic data were scarce and mainly from rats. Chang and coworkers [21] showed that when administered at 6.0 mg/kg p.o. hyperoside could not be detected in plasma of rats either as the unchanged form or as its aglycone or conjugated aglycone form. Observations from the *in vitro* Caco-2 monolayer model and *in situ* intestinal perfusion model indicated that hyperoside has quite limited permeability [25,26]. Juergenliemk and coworkers [26] using three *in vitro* membrane barrier cell systems (Caco-2 cell line, porcine cell cultures of brain capillary endothelial cells and epithelial cells of the plexus chorioidei) demonstrated that the main metabolite of hyperoside, miquelianin (**Fig. 1**), crossed all barriers, being able to reach the CNS after oral administration. In addition, many pharmacological findings on hyperoside were obtained from *in vitro* assays, or topical application, that do not involve systemic metabolism and absorption [14,17].

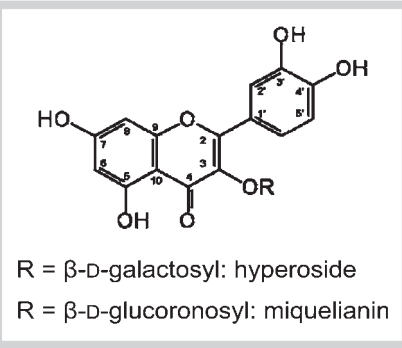

Fig. 1 Chemical structures of hyperoside and miquelianin.

Hyperoside did not induce either mice gross behavior changes or weight loss (data not shown) or deaths up to 40 mg/kg (i.p.) indicating that this compound does not present acute toxicity. In the open field test hyperoside 20 and 40 mg/kg i.p. reduced significantly crossing, rearing and grooming (**Fig. 2**); at 20 mg/kg i.p. it increased mice sleeping time induced by pentobarbital but not the sleep latency (**Fig. 3**). Altogether these results suggest that this compound presents a CNS depressor effect.

Hyperoside is highlighted as one of the active compounds of St John's Wort methanolic crude extract, showing an antidepressant-like effect in the Porsolt's forced swimming test in rats after acute and repeated treatments [6]. In the present study we have reproduced the antidepressant-like effect previously demonstrated by Butterweck and coworkers [6] in rats (1.8 mg/kg/day p.o.) (**Fig. 4**), and also showed the antidepressant-like effect in mice (10 and 20 mg/kg i.p.) (**Fig. 5**). Interestingly, Butterweck and coworkers [6] also observed a miquelianin (1.8 mg/kg/day p.o.) antidepressant-like effect.

The anti-immobility effect of hyperoside was not related to a nonspecific behavioral stimulation, given that it reduced motor activity. This observation corroborates with the hypothesis that the treatment with hyperoside could be antidepressant, since to be considered as a potential antidepressant, a drug must reduce immobility in the FST at doses that do not stimulate locomotion. This result is also in accordance with studies showing that many antidepressants tend to decrease motor activity [21,23] as well as to present sedative effects [27].

Previous studies in search of the mode of action of hyperoside indicated that this flavonoid downregulated circulating plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone, which play an important role in the modulation of hypothalamic-pituitary-adrenal (HPA) axis function, that is altered in patients with major depression [28] and reduced beta2-adrenergic sensitivity in C6 cells, which was demonstrated by the reduction of in-

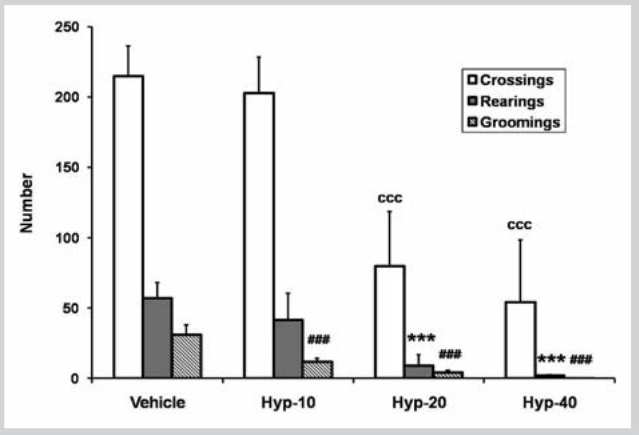

Fig. 2 Effect of hyperoside (10, 20 and 40 mg/kg i.p.) on locomotor activity. Data are presented as mean ± SEM (n = 6–8 mice/group). Significantly different values were detected by one-way ANOVA, crossings $F_{3,27} = 8.92$ ccc $p < 0.001$, rearings $F_{3,27} = 4.70$ *** $p < 0.001$, groomings $F_{3,27} = 9.73$ ### $p < 0.001$, followed by Dunn's post hoc test: compared to vehicle.

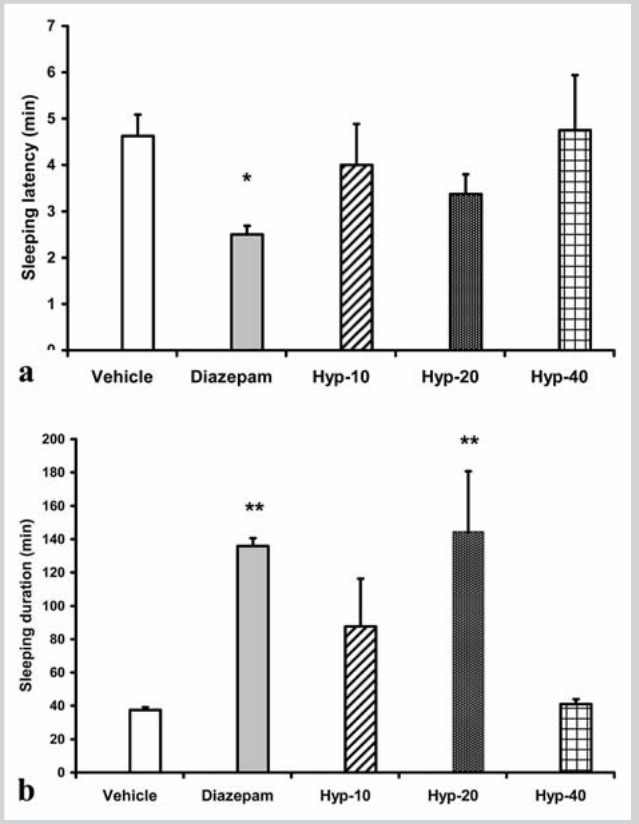

Fig. 3 Effect of hyperoside (10, 20 and 40 mg/kg i.p.) and diazepam (1 mg/kg i.p.) on the potentiation of sodium pentobarbital sleeping latency (**a**) and duration (**b**). Data are presented as mean ± SEM (n = 8 mice/group). Significantly different values were detected by one-way Welch's ANOVA, (**a**) $F_{4,39} = 1.65$ (**b**) $F_{4,39} = 5.88$, followed by Dunnett's post hoc test: * $p < 0.05$.

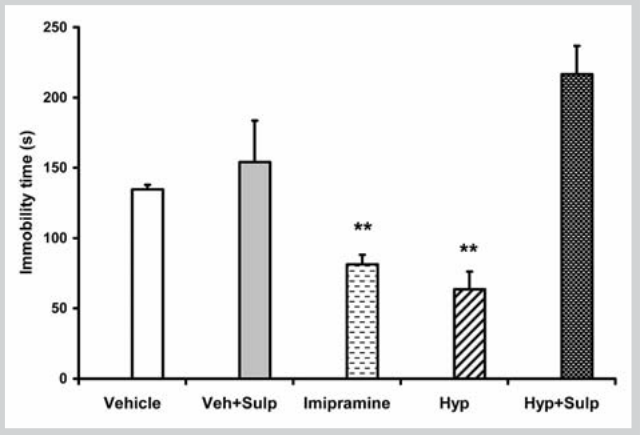

Fig. 4 Effect of hyperoside 1.8 mg/kg/day p.o. on the forced swimming test in rats. Data are presented as mean ± SEM (n = 8–12 mice/group). Significantly different values were detected by one-way Welch's ANOVA $F_{4,47} = 15.55$, followed by Dunnett's post hoc test: * $p < 0.05$.

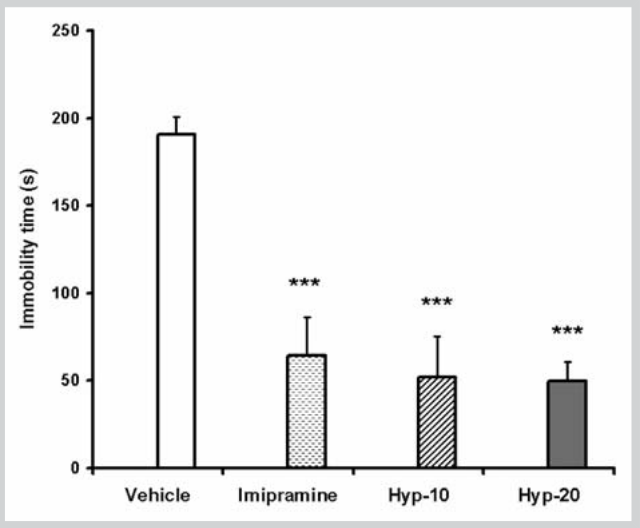

Fig. 5 Effect of hyperoside 10 and 20 mg/kg i.p. on the forced swimming test in mice. Data are presented as mean ± SEM (n = 8 mice/group). Significantly different values were detected by one-way ANOVA $F_{3,31} = 14.92$, followed by Student-Newman-Keuls post-hoc test: *** $p < 0.001$.

tracellular cAMP concentration [29]. Denke and coworkers [30] reported that hyperoside and other flavonoids from *H. perforatum* fairly inhibited dopamine-beta-hydroxylase (DBH) which catalyzes the conversion of dopamine into noradrenaline. This observation is controversial since some reports indicate that the DBH activity is important to the antidepressant effect. DBH blood levels were reduced in mice submitted to chronic mild stress [31], and these alterations were normalized by imipramine treatment. Mice that are unable to synthesize norepinephrine and epinephrine due to targeted disruption of the DBH gene did not respond to several antidepressants in the forced swimming test [32].

In this study we showed that the antidepressant-like effect of hyperoside was prevented by sulpiride (50 mg/kg i.p.), while sulpiride itself did not affect the immobility time (**Fig. 4**). This result indicates that the activation of D2-like receptors accounts for the anti-immobility effect of hyperoside. To our knowledge this is the first evidence that the treatment with hyperoside acts on dopaminergic neurotransmission. Viana and coworkers [9] showed

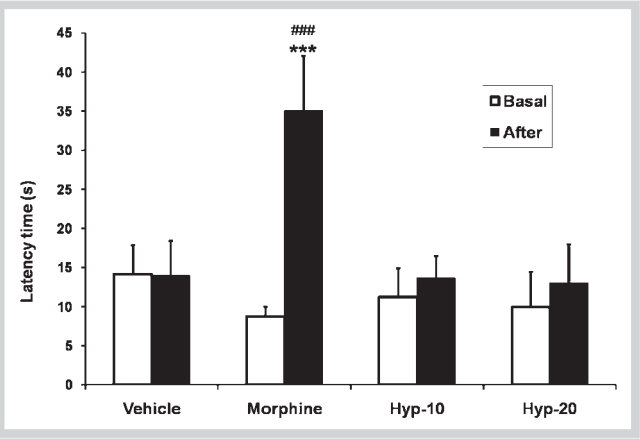

Fig. 6 Effect of hyperoside (10 and 20 mg/kg i.p.) on the hot-plate test. Data are presented as mean ± SEM (n = 6–8 mice/group). Significantly different values were detected by two-way repeated measures ANOVA followed by Student-Newman-Keuls post hoc test: treatment factor $F_{3,59} = 15.21$; latency factor $F_{1,59} = 58.20$; latency vs. treatment factor $F_{3,59} = 37.75$. *** $P < 0.001$ significant difference from vehicle (second latency); ### $p < 0.001$ significant difference from respective basal latency.

Table 1 Effect of hyperoside (20 and 40 mg/kg, p.o.) on the writhing induced by 0.8% acetic acid in mice.

Test samples	Dose (p.o.)	Number of writhings ± S.E.M.	Inhibitory ratio compared to control (%)
Vehicle	10 mL/kg	28.0 ± 4.9	–
Dipyrone	150 mg/kg	9.7 ± 2.5***	65
Hyperoside	20 mg/kg	16.4 ± 3.4	41
Hyperoside	40 mg/kg	20.4 ± 4.2	27

Effect of hyperoside (20 and 40 mg/kg p.o.) on the writhing test. Data are presented in mean ± SEM (n = 13 mice/group). Significantly different values were detected by one-way ANOVA $F_{3,51} = 3.91$ followed by Student-Newman-Keuls post hoc test: *** $p < 0.001$

that the antidepressant-like effect of an *H. caprifoliatum* extract enriched in phloroglucinol derivatives on FST results from an inhibition of neuronal monoamine uptake, mainly dopamine. Dopamine agonists are known to be effective in the FST [33]. The chronic treatment with antidepressants increases D2 receptors functioning [34] and some drugs used to treat human depression act through the dopaminergic system [35]. Altogether these data stress the potential antidepressant effect of *H. caprifoliatum* as well as point to hyperoside as a new chemical feature with dopaminergic properties.

Hyperoside did not display an antinociceptive effect either on the hot-plate (10 and 20 mg/kg i.p.) (**Fig. 6**) or in the acetic acid-induced writhing test (20 and 40 mg/kg p.o.) (**Table 1**). These results are in disagreement with those of Rylski and coworkers [13] who reported antinociceptive properties of hyperoside at lower doses (3.5–10 mg/kg) in the hot-plate test. These conflicting results could be explained by biological and/or inter-laboratory variability or by a bell shaped dose-response curve. By any means, the antinociceptive effect of hyperoside seems to be not robust enough to be reproducible.

In summary, treatment with hyperoside in rats and in mice produces an antidepressant-like effect in FST, a model predictive of antidepressant properties. This effect seems to depend on the D2-like receptor activation. These results strongly suggest that hyperoside has an important contribution to the previously reported antidepressant-like activity of *H. caprifoliatum* [7], along with the phloroglucinol derivatives [9]. Therefore it is in agreement with the concept that the antidepressant effect of *H. perforatum* is due to a variety of constituents rather than to a single compound. Besides, this study indicated that the contribution of hyperoside to the antinociceptive activity of *Hypericum* is uncertain. Other constituents such as benzopyrans, previously reported by our group [12], seem to be more relevant to this effect.

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