Flavonoids from *Hypericum perforatum* Show Antidepressant Activity in the Forced Swimming Test

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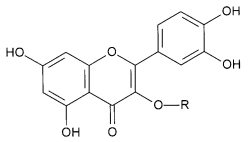
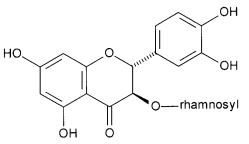
Abstract: **It has been shown recently that a flavonoid fraction (fraction II) obtained from a crude extract of *Hypericum perforatum* (St. John's wort) was remarkably active in the forced swimming test (FST). Fraction II was further separated using MLCCC to give fractions IIa and IIb. Both fractions proved to be active in the FST at different dosages. Further separation of fraction IIa by preparative HPLC yielded fraction IIa_1 which mainly was composed of hyperoside, isoquercitrin, miquelianin and quercitrin, and fraction IIa_2 which contained small amounts of hyperoside and astilbin, while most compounds were not known. Both fractions were active after acute treatment in the FST. Isolates obtained from these fractions including hyperoside, isoquercitrin, quercitrin, miquelianin, the aglycone quercetin and astilbin, were tested for activity in the FST. Except for quercetin, quercitrin and astilbin all compounds were active. To exclude false positive results in the FST the validity was checked in open field experiments and in the FST after 12 days of daily treatment.**

While extracts prepared from the flowering upper parts of *Hypericum perforatum* L. (Clusiaceae) belong to the clinically most investigated plant extracts and are successfully used as an antidepressant drug, the constituents responsible for their efficacy are still a matter of debate. The crude drug contains a wide range of compounds such as phenylpropanes, flavonol derivatives, biflavones, proanthocyanidins, xanthones, phloroglucinols, some amino acids, naphthodianthrones and essential oil constituents (1). Recently, the antidepressant activity of *Hypericum* extracts has been attributed to the naphthodianthrones hypericin and pseudohypericin (2), (3) and to the phloroglucinol derivative hyperforin (4), (5). However, a fraction which contained mainly flavonoids [fraction II in (2)] was significantly active in Porsolt's Forced Swimming Test (FST) (6) in doses comparable to the crude extract with U-shaped dose response curves (2). This fraction was further investigated for active constituents in the present paper.



R = H: Quercetin
R = galactosyl: Hyperoside
R = glucosyl: Isoquercitrin
R = glucuronosyl: Miquelianin
R = rhamnosyl: Quercitrin



Astilbin

Fractionation of fraction II (2) by means of MLCCC yielded fractions IIa and IIb. For pharmacological experiments the doses were chosen on the basis of the amount in the original fraction II (see Scheme of application in Methods and Materials) and imipramine was used as a positive control. As shown in Figure **1**, fraction IIa significantly reduced immobility time in the FST at doses of 5 and 10.05 mg/kg, whereas fraction IIb was active at a dose of 3.5 mg/kg. As shown by TLC, fraction IIa was composed of hyperoside (quercetin 3-*O*-galactoside), quercetrin (quercetin 3-*O*-rhamnoside), isoquercitrin (quercetin 3-*O*-glucoside) and unidentified compounds, whereas

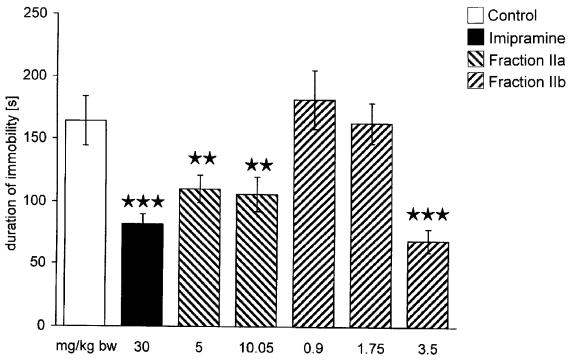


Fig. 1 Activity of fractions IIa and IIb in the forced swimming test. According to Porsolt (6) substances were administered orally 24, 5 and 1 h before the test (n = 7 – 10; ★★★ $p < 0.001$ vs. control; ★★ $p < 0.01$ vs. control).

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fraction IIb contained mainly hyperoside, quercitrin and unidentified compounds. Therefore, fraction IIa, which was 85% of fraction II, was further fractionated by preparative HPLC. Fraction IIa_1 contained mainly hyperoside, isoquercitrin, quercitrin and quercetin 3-*O*-glucuronide (miquelianin; not yet known for *H. perforatum*); fraction IIa_2 contained low amounts of hyperoside and astilbin (taxifolin 3-*O*-rhamnopyranoside) that is also a new flavonoid for *H. perforatum*. Both fractions significantly reduced immobility time in the FST (Fig. **2**).

Because hyperoside was one of the main constituents in all fractions obtained it was first tested in the FST for its antidepressant activity. The concentration of hyperoside for pharmacological experiments was chosen on the basis of its amount in fraction II [(2), see Scheme of application]. Hyperoside was active in the FST after acute treatment at doses of 0.6 and 1.3 mg/kg, whereas concentrations below and above this were not significant or inactive (Fig. **3**). This indicates an inverted U-shaped activity for hyperoside as was already observed for the entire crude extract of *H. perforatum*, some fractions thereof (2) and hypericin and pseudohypericin (3).

These results demonstrate that hyperoside is not the only active substance in fraction II, because fractions II, IIa and IIa_1 clearly exceeded the effects of hyperoside in the FST. Hence, astilbin and miquelianin isolated from fraction IIa, and commercially available quercitrin and isoquercitrin were tested in the FST in comparison to the aglycone quercetin. Substances were administered orally at doses corresponding to the active doses of hyperoside. While astilbin and quercitrin were inactive in the FST, miquelianin as well as isoquercitrin significantly reduced immobility time at a dosage of 0.6 mg/kg (Fig. **4**). The aglycone quercetin was inactive when administered at a dose of 0.4 mg, analogous to 0.6 mg/kg hyperoside (values not shown).

As changes in immobility may be due to changes in locomotor activity caused by central stimulating agents [e.g., induced hypermotility after treatment with amphetamine or caffeine (7)], animals were supplementarily tested in an open field test (8). Hyperoside, miquelianin and isoquercitrin were administered orally 24 h, 5 h and 1 h before the test. The animals were then placed individually into the open field and the number of line-crossings within 5 min was recorded. At a dose of 0.6 mg/kg, which significantly reduced immobility time in the FST, no changes of locomotor activity were observed. This result confirms the assumption that the antidepressant effects of the flavonol glycosides in the FST are specific.

Drugs acting as antihistaminics give false positive results in the FST, but will loose their activity after repeated treatment (7). Hence, hyperoside, miquelianin and isoquercitrin were administered at a dose of 0.6 mg/kg, imipramine at a dose of 20 mg/kg once daily for twelve days. Control animals received distilled water. After this period the FST was performed again. As shown in Figure **5**, hyperoside as well as miquelianin still significantly decreased immobility time, whereas isoquercitrin was less active.

Previous reports suggest that flavonoids are part of the constituents responsible for the efficacy of *Hypericum* extracts but without clear cut results: a flavonoid-containing fraction *in vitro* inhibited monoamine oxidase (9) and catechol-*O*-methyl transferase (10). While the crude *Hypericum* extract interferred with the binding of ligands to the benzodiazepine receptor as well as the μ and κ opioid receptors, hyperoside, quercitrin and rutin as well as quercetin, kaempferol and quercitrin did not (11), (12). The results obtained here clearly show that three quercetin 3-*O*-glycosides of *Hypericum* extract, the galactoside, the glucoside and the glucuronide, are active compounds in the FST, whereas the rhamnoside includ-

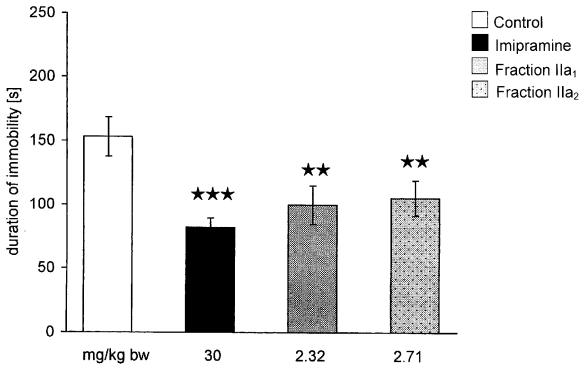

Fig. 2 Fraction IIa_1 as well as fraction IIa_2 reduces the immobility in the forced swimming test. Substances were administered 24, 5 and 1 h before the test (n = 8–10; ★★★ $p < 0.001$ vs. control; ★★ $p < 0.01$ vs. control).

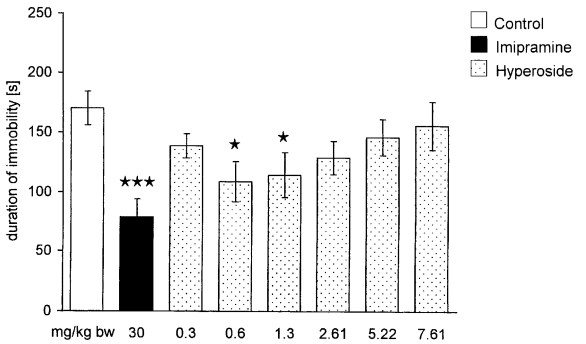

Fig. 3 Activity of hyperoside in the forced swimming test after acute treatment (24, 5 and 1 h before the test; n = 9–12; ★★★ $p < 0.001$ vs. control; ★ $p < 0.05$ vs. control).

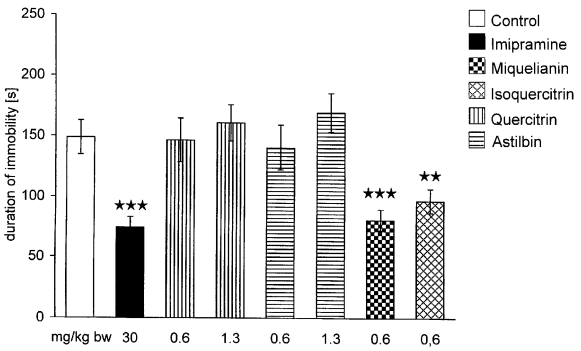

Fig. 4 Activity of quercitrin, astilbin, miquelianin and isoquercitrine in the forced swimming test (24, 5 and 1 h before the test; n = 7–9; ★★★ $p < 0.001$ vs. control; ★★ $p < 0.01$ vs. control).

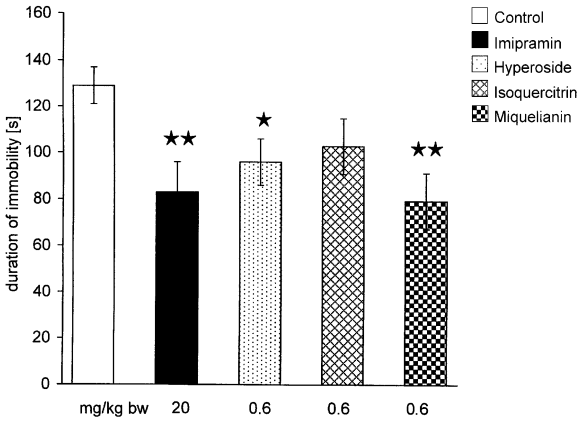

Fig. 5 Activity of hyperoside, isoquercitrin and miquelianin the FST after 12 days of daily treatment (n = 8; ★★ $p < 0.01$ vs. control; ★ $p < 0.05$ vs. control).

ing the 2,3-dihydroquercetin 3-*O*-glycoside astilbin and the aglycone quercetin were not. In recent investigations (2) fractions containing high amounts of quercetin 3-*O*-rutinoside (rutin) showed no activity in the FST indicating that rutin is one of the non-active flavonol glycosides. Thus, it seems that the sugar part of the flavonol aglycone influences activity in the FST; this hypothesis as well as the mode of action of the flavonol glycosides will be further investigated.

Materials and Methods

Plant material: For all experiments, a lyophilized methanolic native extract (drug/extract ratio: 4 – 7 : 1) manufactured by Lichtwer Pharma AG, Berlin, was used. Analytical data were published in (2). A voucher is deposited at the Institute of Pharmaceutical Biology and Phytochemistry under GJ 160.

MLCCC: Fraction II was separated into fractions IIa and IIb by MLCCC with a Multilayer Coil Separator (13) [P.C. Inc. Potomac, Maryland, USA] using $CHCl_3$/MeOH/H_2O (13 : 7 : 4) with the upper phase as mobile phase and the lower phase as the stationary phase; column: 375 ml; rotation 800 rpm; flow rate: 1.0 ml/min. Fraction IIa (elution volume 0 – 60 ml) represented 85% of fraction II with a total flavonoid content of 44.2% (according to Deutsches Arzneibuch 1998, monography Crataegus folium cum flore) and hyperoside, quercitrin and isoquercitrin as flavonol glycosides as detected by TLC (silica gel; EtOAc/HCOOH/H_2O 30 : 2 : 3; Naturstoff-reagent for detection). Fraction IIb (60 – 100 ml and the stationary phase) was 15% of fraction II with a total flavonoid content of 43.6% and hyperoside and quercitrin (TLC).

HPLC: Fractions IIa_1 and IIa_2 were obtained by preparative HPLC. Column: Knauer RP-18, Eurospher 100, 7 μm (205 × 20 mm); flow: 6.0 ml/min; injection volume: 800 μl. Solvent gradient was composed of CH_3CN (A) and H_2O with 0.1% TFA (B): 0 – 20 min 30 – 35% A, 20 – 25 min: 35 – 100% A, 25 – 35 min: 100% A, 35 – 40 min: 100 – 30% A. Equipment: Waters 515 with a Waters Lambda-max Model 481 LC spectral photometer. Fraction IIa_1 represented two main signals (12.5 min and 15.8 min) which showed flavonoid spectra; both signal fractions were cut off and combined; fraction IIa_1 was 46% of fraction IIa with 96.5% total flavonoids and hyperoside, quercitrin, isoquercitrin and miquelianin as detectable constituents (TLC). Fraction IIa_2 represents the residual 54% of fraction IIa with 8.5% total flavonoids and hyperoside and astilbin as detectable flavonoids (TLC). Isolation and identification of miquelianin and astilbin are to be published (Jürgenliemk et al., in prep.).

Animals: Male CD rats (Charles River WIGA, Sulzfeld, Germany) were used. The animals were kept in a 12 h light/dark cycle at a constant temperature of 25 ± 1 °C with free access to food (altromin 1324) and tap water. Animal experiments were officially approved by the Regierungspräsident, Münster (A 38/93).

Substances: Imipramine-HCl (positive control) was purchased from Sigma (Deisenhofen, Germany), hyperoside, quercitrin, and quercetin were purchased from Roth (Karlsruhe, Germany). Astilbin, miquelianin and isoquercitrin were isolated during a study on the flavonoids of *H. perforatum* extract (Jürgenliemk et al.; in prep.).

Drug treatment: In the acute and in the chronic experiments all substances were administered orally using the gavage technique; all substances were dissolved in deionized water, the control animals received deionized water only. For the acute study all substances were administered orally 24 h, 5 h and 1 h before the test (7). The chronic treated rats received the drugs once daily.

Measurement of locomotor activity: For measurement of locomotor activity naive rats were placed into an open field apparatus 1 h after the last drug treatment. Locomotor activity was determined by counting the number of line crossings within a period of 5 min. No significant changes were observed between control, the imipramine group and the hyeroside, the miquelianin and the isoquercitrin groups.

Measurement of antidepressant activity was performed using the Forced Swimming Test according to the method published by Porsolt (6). Male rats were placed into a plexiglass cylinder (40 × 18 cm i.d.) containing a column of 17 cm of water at 25 ±

Scheme of application:

Fraction	administered dose [mg/kg b.w.]	corresponding to fraction II [mg/kg b.w.]	corresponding to *Hypericum* extract [mg/kg b.w.]
IIa	5.03	5.9	120
	10.05	11.8	240
IIb	0.88	5.9	120
	1.75	11.8	240
	3.5	23.6	480
IIa_1	2.32	5.9	120
IIa_2	2.71	5.9	120
Hyperoside	0.3	0.75	15
	0.6	1.5	30
	1.3	2.95	60
	2.6	5.9	120
	5.22	11.8	240
	7.61	17.2	350

1 °C. According to Porsolt the rats learned in a pretest session of 15 min that they could not excape from the cylinder. In the test period, 24 h later, the animals were exposed to the experimental conditions for 5 min.

All behavioural studies were performed between 1 – 3 p.m. and recorded by a video camera. The tapes were evaluated afterwards by an observer who was not informed about the kind of treatment each animal had received.

A dosage of 30 mg/kg imipramine was chosen for the acute study because former experiments indicated that dosages below 30 mg were only partially active; for chronic treatment 20 mg/kg proved to be an active dosage without toxic effects, whereas higher doses of imipramin showed toxic effects in the chronic treatment.

Statistics: All data were expressed as the mean ± SE. Group mean differences were ascertained with analysis of variance (ANOVA). Multiple comparisons among treatment means were checked with the LSD (least significant determination). The results were considered significant if the probability of error was < 5 %.

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