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# St. John's wort flavonoids and their metabolites show antidepressant activity and accumulate in brain after multiple oral doses

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Over the last few years many data have been published suggesting a participation of quercetin flavonoids in the antidepressive effect of St. John's wort (SJW) extract. To elucidate these data more deeply we performed two animal behavioural studies examining the antidepressant effects of SJW extract, rutin and, in addition, the quercetin metabolite isorhamnetin. The substances were in all cases compared to imipramine using Porsolt's forced swimming test (FST) after oral gavage of the substances over a 9 day period. All three compounds were found to be effective, with isorhamnetin exhibiting the strongest effect. In addition to this pharmacological study, we carried out two pharmacokinetic studies to examine the CNS level time-curve of the quercetin flavonoids after a single oral dose of SJW extract (1600 mg/kg) and isoquercitrin (100 mg/kg), respectively, and to observe the cumulative effects after daily repeated oral doses of SJW extract over 8 days. After a single dose the maximal CNS levels for quercetin (340 ng/g) and isorhamnetin/tamarixetin (50 ng/g) were found at 4 h. With repeated doses the maximal cumulation for quercetin (367 ng/g) occurred after 5 days whilst isorhamnetin/tamarixetin (640 ng/g) did not reach its maximal cumulation level within the 8 day test period.

## 1. Introduction

Depression is a common mental disorder with an estimated lifetime prevalence rate of about 17% (Lepine et al. 1997). *Hypericum perforatum* (St. John's wort, SJW) is the only herbal alternative to synthetic antidepressants in the therapy of mild to moderate depression. The effectiveness of alcoholic SJW extracts has been demonstrated in several clinical studies (Linde et al. 2005). Moreover, these extracts are characterised by a favorable side-effect profile. The number of prescriptions issued for SJW preparations in Germany and the USA attests to its popularity in these countries (e.g. 100 million defined daily doses in Germany alone in 2003) (Schwabe and Paffrath 2004).

SJW extracts contain a wide spectrum of different components, including phloroglucinoles, flavonoids, naphthodianthrones, proanthocyanidines, xanthones, some amino acids and essential oils.

Since clinical studies have demonstrated the efficacy of SJW extracts in the treatment of mild to moderate depression, there has been increased interest in identifying the pharmacologically active components. A large number of *in vitro* and *in vivo* studies indicate, that the antidepressant activity of the SJW extract is not related to any single compound or any single class of compounds. In all probability the pharmacological effect of the extract results from different synergistically acting compounds.

Studies suggest the involvement of hyperforin in the activity of the extracts by different mechanisms (Bhattacharya et al. 1998; Roz and Rehavi 2003; Singer et al. 1999; Wurglics and Schubert-Zsilavecz 2006). The most interesting mode of action, comparable to that of synthetic antidepressants, is the uptake inhibition of the neurotransmitters 5-HT (Schulte-Löbbert et al. 2004), norepinephrine (NA), and dopamine (DA) (Müller et al. 1997). The inhibition occurs in a non-specific manner, with GABA and L-glutamate uptake also being inhibited. These effects are probably a result of an increase in the intracellular $Na^+$ concentration, due to an alteration of sodium conductive pathways (Gobbi et al. 2001; Müller et al. 2001; Singer et al. 1999; Wonnemann et al. 2000) and/or to a modified neurotransmitter storage in synaptic vesicles (Gobbi et al. 1999; Roz and Rehavi 2003).

The antidepressive potential of the naphthodianthrone derivatives hypericin and pseudohypericin was shown using behavioural tests. In the forced swimming test (FST) hypericin reduced the immobility time significantly after a 14 day treatment (Butterweck et al. 2001) or after acute treatment when given concurrently with procyanidins or flavonoids (Butterweck et al. 2003). The mode of action of the naphthodianthrones is still unclear. Recent studies point to a decrease in the functional activity of the HPA (hypothalamic-pituitary-adrenal) axis (Butterweck et al. 2001).

Even though pure hyperforin as well as pure hypericin are active in behavioural models such as the FST; Butterweck et al. (2003) were able to show, that the removal of both substances from a SJW extract did not induce a significant

reduction in efficacy. In the same study, a SJW extract, containing considerable amounts of flavonoids, but being free from hyperforin and hypericin, demonstrated that the results of the FST were comparable to those employing the genuine extract. These findings correlate with the examination of different isolated flavonoids in depression relevant animal models. Butterweck et al. (2000) also described antidepressant-like activities in the FST for hyperoside, miquelianin and isoquercitrin. In addition and in contrast to the removal of hyperforin and hypericin, the fact that rutin is essential for the activity of SJW extracts was demonstrated by the FST in rats. Extracts without rutin, or with a very low rutin content, are completely inactive in this test system. Activity was restored when rutin was added to the extract (Nöldner and Schötz 2002).

These results prompted us to re-evalute the pharmacological properties of rutin in the animal model. Additionally, we included the known flavonoid metabolite isorhamnetin in our investigations and we also examined the plasma and CNS pharmacokinetics of the SJW flavonoids. To date only one study concerning the pharmacokinetic profile of *Hypericum perforatum* flavonoids in human plasma

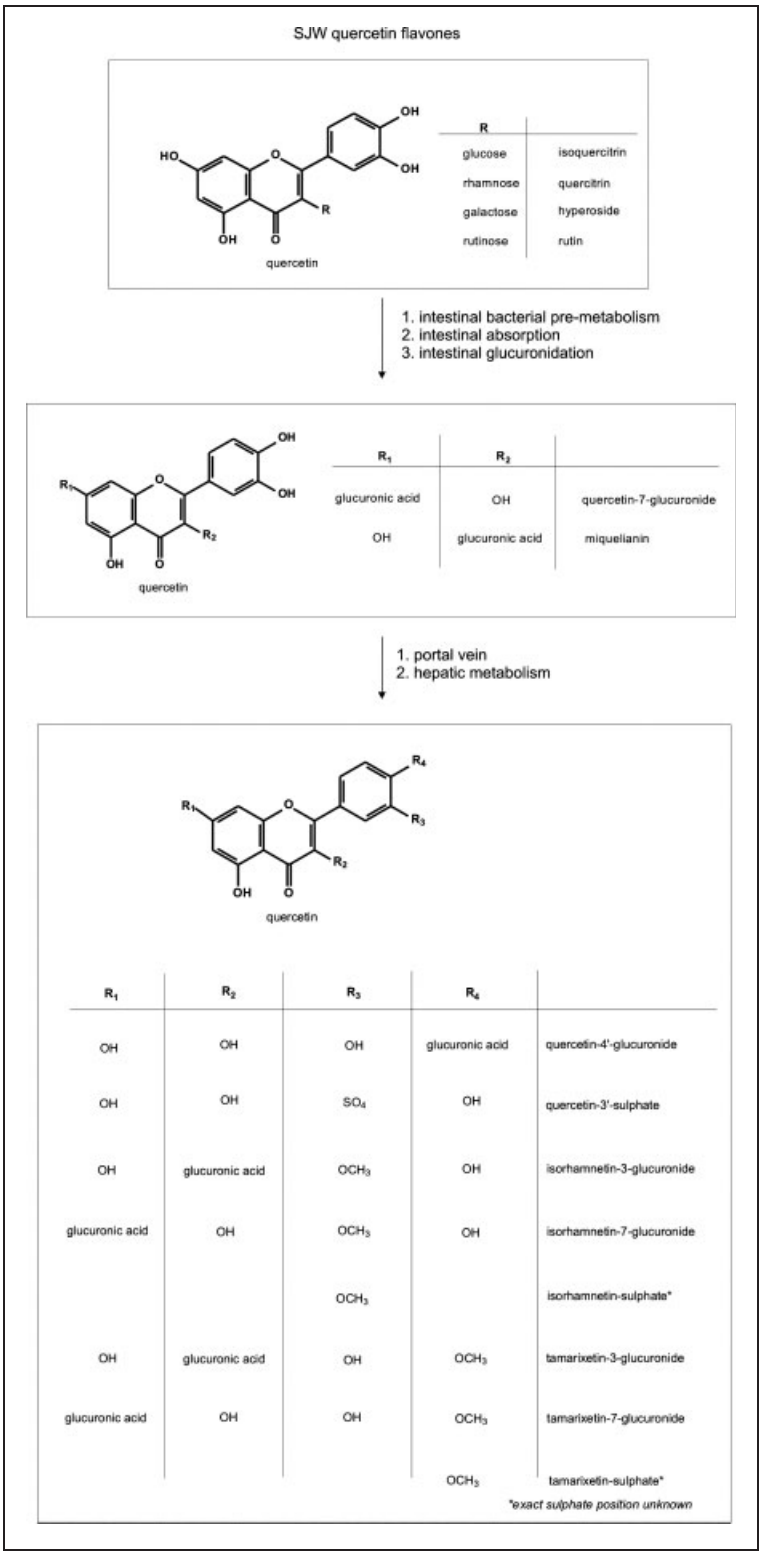


Fig. 1:
Structure of the SJW quercetin flavonoids including its two step metabolism in intestinal cells and liver

has been published (Schulz et al. 2005). Further pharmacokinetic data are available for rutin, both pure and from buckwheat tea, for the aglycone quercetin and for other flavonoid glycosides (administered in the form of onions) (Day et al. 2001; Erlund et al. 2000; Graefe et al. 2001; Manach et al. 1997). These investigations showed that the original flavonoids as well as free aglycones (Cermak et al. 2003) could not be detected in plasma after oral administration although different metabolites arising from these compounds were found (O'Leary et al. 2003). Figure 1 gives a short overview about SJW flavonoids and their metabolic pathway. The bioavailability of SJW flavonoids and their metabolites in the CNS – the site of action for an antidepressant – has been established recently in our laboratories using rats as experimental animals (Paulke et al. 2006).

## 2. Investigations and results

### *2.1. Pharmacological studies*

Oral administration of SJW extract WS® 5572 over a time period of 9 days decreased significantly the duration of immobility in the FST, measured one hour after the last drug administration. This effect was dose dependent with a maximal effect after 400 mg/kg b.w. (Fig. 2). The efficacy of 200 mg/kg SJW extract or more is comparable to that of the tricyclic antidepressant imipramine (30 mg/kg) (results not shown).

Administration of the SJW constituent rutin also decreased the immobilization time. A strong and statistical significant effect was observed after daily administration of 12 mg/kg or more. Increasing the dosage up to 48 mg/kg shortened the immobilization time to the same level as 30 mg/kg imipramine (Fig. 3).

The strongest efficacy in the FST was seen after administration of the flavonoid metabolite isorhamnetin. Daily administration of 3 mg/kg induced a statistical significant decrease of the immobility time. Increasing the dosage up to 10 mg/kg intensified this effect significantly. At both dosages isorhamnetin is more effective than the tricyclic antidepressant imipramine which was given for comparison (Fig. 4).

In order to prove that the reduction of immobility in the FST is not caused by CNS stimulation, all tested extracts and constituents were also studied in a motility test system, which measured the motor activity of the rats. None of the extracts or constituents influenced in the dosage used the motility of the animals. This indicates that the effects in the FST are not affected by stimulating locomotor activity per se.

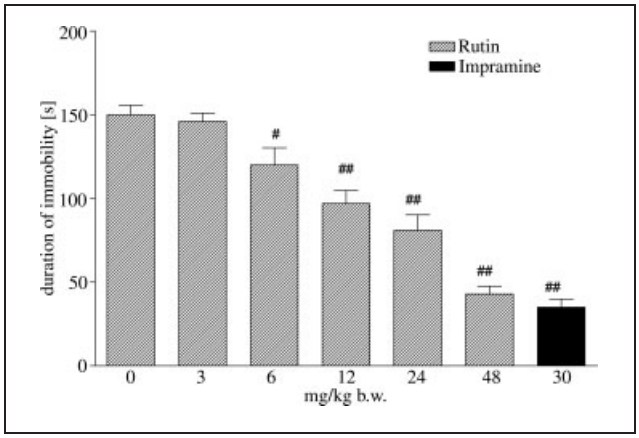


Fig. 3: Effect of rutin versus imipramine in Porsolt's FST. (## $P > 0.99$, # $P > 0.95$)

### *2.2. Pharmacokinetic studies*

Previous pharmacokinetic studies in humans and animals indicated that, *in vivo*, only traces of unconjugated quercetin (Cermak et al. 2003; Day et al. 2001; Wittig et al. 2001) or intact quercetin glycosides (Erlund et al. 1999; Graefe et al. 2001) are present. During absorption nearly all quercetin glycosides were deglycosylated and further metabolized through glucuronidation, sulfatation and/or methylation so that numerous metabolites appear in plasma and tissue (Graefe et al. 2001; Manach et al. 1997; O'Leary et al. 2003; Wittig et al. 2001). By acid hydrolysis, it is possible to obtain the respective aglycones quercetin, tamarixetin and isorhamnetin which could be easily analyzed by a HPLC fluorescence method (Paulke et al. 2006).

#### *2.2.1. Study 1*

In Fig. 5 we present the plasma levels of quercetin and isorhamnetin/tamarixetin after administration of SJW extract or pure isoquercitrin.

Following a single dose administration of SJW extract, the quercetin plasma level increased rapidly and reached the maximum of about 700 ng/mL after 4 h which was followed by a slow, continuous decrease over the next 44 h.

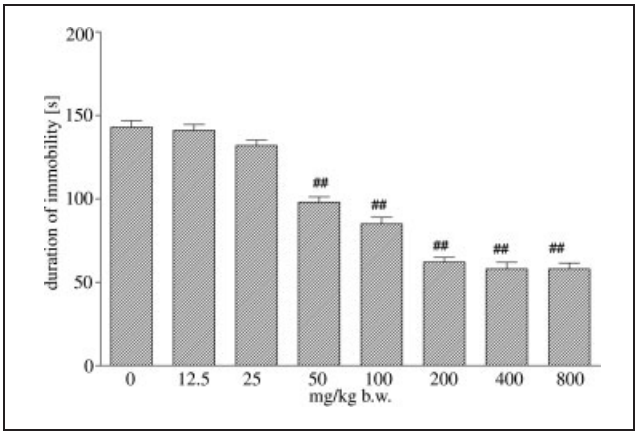


Fig. 2: Effect of SJW extract WS® 5572 on the immobilisation time in Porsolt's FST (## $P > 0.99$)

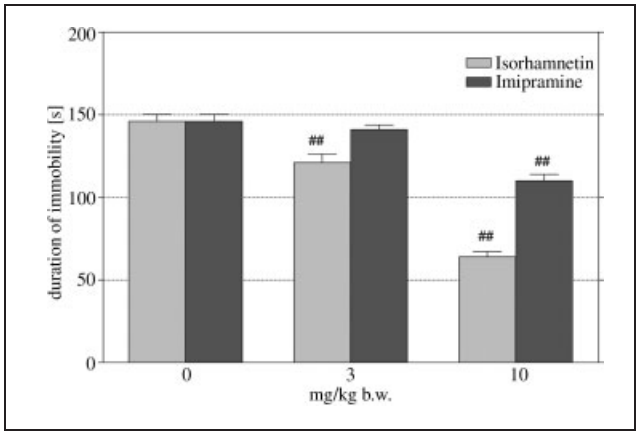


Fig. 4: Effect of isorhamnetin versus imipramine in Porsolt's FST. (## $P >$ 0.99)

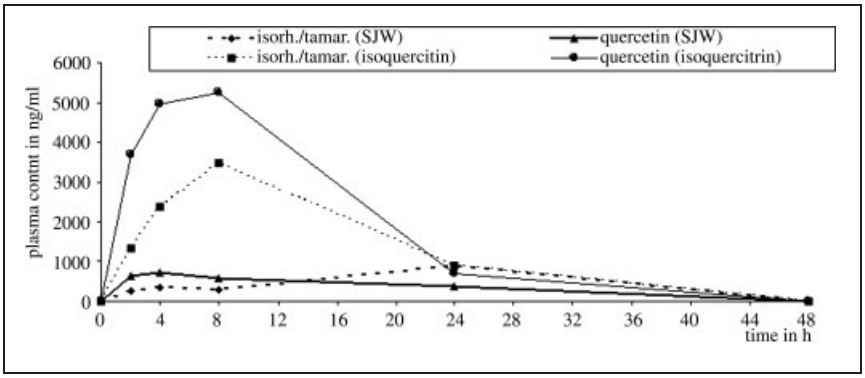


Fig. 5:
Plasma concentration time curves of quercetin and isorhamnetin/tamarixetin after administration of SJW extract or pure isoquercitrin

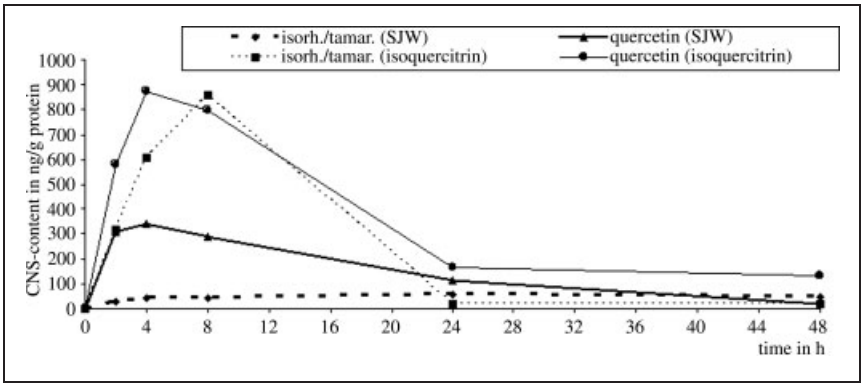


Fig. 6:
CNS concentration time curves of quercetin and isorhamnetin/tamarixetin after administration of SJW extract or pure isoquercitrin

After 24 h, 50% of $C_{max}$ was still measurable. These data are in good agreement with the investigations by Schulz et al. (2005), who found two maxima in a more detailed curve, the first after about 2 h and a second after 6 h. The two maxima may be explained by different concentration-time curves for monogylcosides (e.g. isoquercitrin, hyperoside) and diglycosides (e.g. rutin) as suggested by Erlund et al. (2000) and Graefe et al. (1999).

The shape of the concentration vs. time curve for isorhamnetin/tamarixetin in plasma was completely different from that for quercetin. The concentration level increased much slower and reached the maximum after 24 h ($C_{max} = 903$ ng/mL), nevertheless the $C_{max}$ was comparable to that of quercetin. This delayed maximum might be due to an additional metabolization step, the formation of 3′-*O*-methylquercetin (isorhamnetin) and 4′-*O*-methylquercetin (tamarixetin) in the liver by COMT (O'Leary et al. 2003).

In the control group (oral dose 100 mg/kg isoquercitrin) the quercetin and isorhamnetin/tamarixetin concentrations in plasma were much higher than in the SJW extract group. Most interesting was that the shapes of the curves concentration vs. time for quercetin in plasma were similar in both groups, but those for isorhamnetin/tamarixetin were markedly different. Whereas in the SJW extract group the maximal plasma concentration of isorhamnetin/tamarixetin occurred at 24 h, in the isoquercitrin group the blood concentrations increased rapidly ($t_{max} = 8$ h) and began to decline after the first maximum.

The curves concentration vs. time in CNS are summarized in Fig. 6. For quercetin the profile seems to be congruent with the plasma curves. The maximum brain concentration appeared after 4 h and reached approx. 340 ng/g protein and 870 ng/g protein for SJW extract and isoquercitrin, respectively. After 24 h only very low levels could be detected, but these were not quantifiable. For isorhamnetin/

**Table 1: CNS and plasma levels after isoquercitrin and SJW extract treatment**

| Time in h after isoquercitrin or ① SJW extract ② administration | | CNS | | Plasma | |
|---|---|---|---|---|---|
| | | Quercetin in ng/g protein | isorhamnetin/tamarixetin in ng/g protein | Quercetin in ng/mL | isorhamnetin/tamarixetin in ng/mL |
| ① | 2 | 582.04 ± 150.56 | 317.77 ± 133.95 | 3691.62 ± 1951.13 | 1340.85 ± 394.55 |
| | 4 | 871.12 ± 234.25 | 610.24 ± 89.45 | 4959.89 ± 1584.56 | 2374.11 ± 407.73 |
| | 8 | 796.28 ± 162.67 | 858.86 ± 238.95 | 5251.35 ± 1345.50 | 3502.68 ± 990.92 |
| | 24 | 166.88 ± 43.68 | < LOQ – | 701.32 ± 321.64 | 898.91 ± 404.41 |
| | 48 | 130.74 ± 38.86 | < LOQ – | < LOQ – | < LOQ – |
| ② | 2 | 308.83 ± 29.59 | 27.90 ± 11.64 | 624.50 ± 256.11 | 248.17 ± 78.74 |
| | 4 | 341.20 ± 55.32 | 42.84 ± 19.03 | 727.05 ± 262.29 | 346.84 ± 151.70 |
| | 8 | 286.01 ± 26.00 | 43.44 ± 25.97 | 562.54 ± 242.89 | 281.27 ± 151.97 |
| | 24 | 113.99 ± 35.47 | 55.72 ± 23.37 | 378.97 ± 150.14 | 903.17 ± 327.58 |
| | 48 | < LOQ – | 46.38 ± 33.27 | < LOQ – | < LOQ – |

LOQ = Limit of quantification
Concentrations in brain are given as ng/g protein and in plasma as ng/ml

**Table 2: Brain and plasma levels after repeated doses of SJW extract**

| Source of flavonoids | t [d] | CNS | | Plasma | |
|---|---|---|---|---|---|
| | | Quercetin [ng/g] | Isorhamnetin/ tamarixetin [ng/g] | Quercetin [ng/mL] | Isorhamnetin/ tamarixetin [ng/mL] |
| Standard diet | | 207.57 ± 18.84 | < LOQ − | 159.48 ± 64.9 | 29.38 ± 10.5 |
| SJW extract | 1 | 247.11 ± 84.6 | 138.24 ± 56.6 | 569.90 ± 312.8 | 492.39 ± 232.1 |
| | 5 | 366.79 ± 92.2 | 494.08 ± 134.2 | 2612.84 ± 1248.7 | 1745.82 ± 966.2 |
| | 8 | 383.98 ± 111.3 | 640.09 ± 154.6 | 2904.24 ± 1144.4 | 2350.46 ± 866.9 |
| Flavonoid free diet | | < LOQ | < LOQ | < LOQ | < LOQ |

Concentrations in brain are given as ng/g protein and in plasma as ng/ml.

tamarixetin the curves show different profiles depending on the treatment. Under SJW treatment, the isorhamnetin/tamarixetin brain levels reached less than a fifth compared to the quercetin brain levels, but after reaching $C_{max}$ the levels remained constant for at least 44 h. Administration of isoquercitrin led to maximal brain levels of quercetin and isorhamnetin/tamarixetin of about 870 ng/g protein after 4 h and 8 h, respectively, and in contrast to the SJW group the isorhamnetin/tamarixetin level decreased within 20 h (Table 1).

In parallel, we checked flavonoid plasma and CNS levels in animals receiving neither SJW extract nor isoquercitrin, but a standard diet or a flavonoid free diet (see Table 2).

### *2.2.2. Study 2*

The results of study 1, especially the slow decrease of isorhamnetin/tamarixetin in plasma and brain tissue, induced us to conduct a multiple dose study to examine a possible accumulation of flavonoid metabolites in plasma and brain. Three groups of rats received an oral daily dose of 1600 mg/kg SJW extract for 1, 5, and 8 days, respectively. After these times, plasma and brain samples were obtained, by decapitating the rats 4 h after the last application. As seen in study 1 quercetin and isorhamnetin/tamarixetin levels can still be detected after a time lapse of 24 h. With a repeated daily dose, an accumulation of the analytes would be expected. As shown in Fig. 7, the plasma levels of isorhamnetin/tamarixetin increased continuously from day 1 to day 8, while quercetin accumulation in plasma was significant only from days 1 to 5, and after that time concentration appears to remain constant. The concentrations of both, quercetin and isorhamnetin/tamarixetin, increased about five-fold within 8 days. Interestingly, only isorhamnetin/tamarixetin is likely to accumulate in brain (see Fig. 8), its level is increased from day 1 to day 8 by the same magnitude as in plasma. For quercetin almost no accumulation could be observed.

## 3. Discussion

Until now the significance of *Hypericum* flavonoids in the clinical effectiveness of SJW extract is still under discussion. There are hints for a potential correlation between quercetin flavonoid content and a catecholamine-*O*-methyl-transferase (COMT)/monoaminoxidase (MAO)-inhibition (Singh et al. 2003). Another study investigated receptor binding profiles of SJW flavonoids and found some significant effects for rutin ($\alpha_{2C}$, M5), hyperoside ($\alpha_{2A}$) and miquelianin ($\alpha_{2C}$, M2, M5) (Butterweck et al. 2002). However, these effects are rather weak and cannot explain the antidepressive effect of SJW flavonoids.

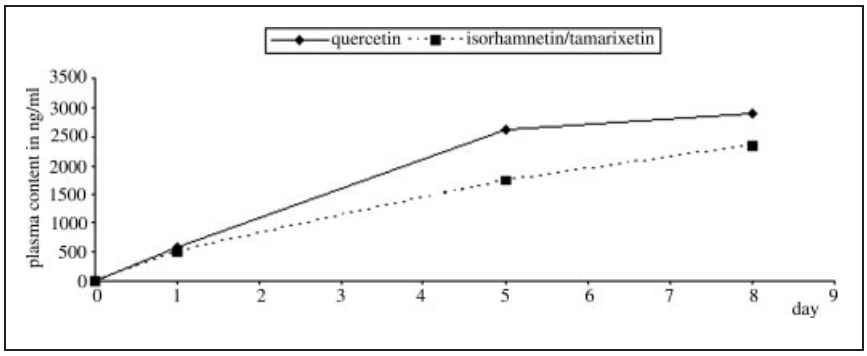


Fig. 7:
Quercetin and isorhamnetin/tamarixetin plasma concentration time curves after 8 day treatment with SJW extract

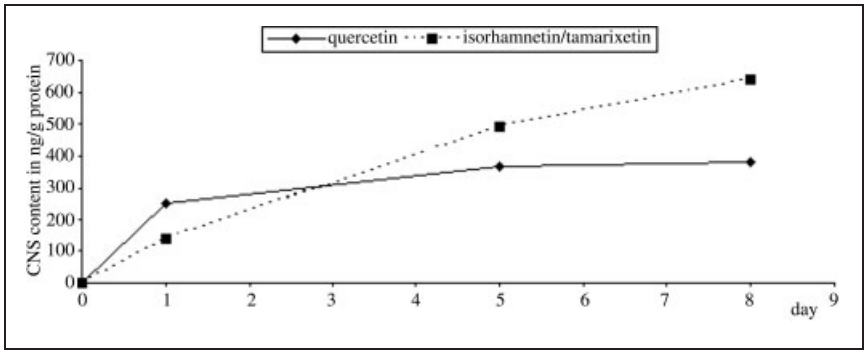


Fig. 8:
CNS concentration time curves after 8 day treatment with SJW extract

Even though no molecular mechanism has been defined, several *in vivo* studies strongly suggest the participation of flavonoids in the antidepressive activity of SJW extract using Porsolt's FST (Nöldner 2001; Nöldner and Schotz 2002). Interestingly only the quercetin flavonoids hyperoside, isoquercitrin, miquelianin and the biflavonoid I 3, II 8-biapigenin showed a statistically significant ($p < 0.05$) reduction of immobility time. For quercitrin and the aglycone quercetin no significant effect was measurable. This outcome is astonishing considering the common metabolism of SJW quercetin flavonoids. After oral uptake they are deglycosylated in the small intestine and, after absorption, the quercetin aglycone is glucuronidated in different positions to yield various metabolites (e.g. miquelianin). Further methylation in the liver at position 3′ or 4′ is possible leading to isorhamnetin and tamarixetin (Day et al. 2001; O'Leary et al. 2003). Based on these findings, it seems unlikely that genuine *Hypericum* flavonoid glycosides reach the plasma or tissues (Cermak et al. 2003; Graefe et al. 2001). Various studies documented a satisfactory absorption of the flavonoids, including rutin (Erlund et al. 2000; Manach et al. 1997). Consequently it is difficult to explain why there should be serious differences in the effect of quercetin flavonoids on the FST.

In addition, a study of Nöldner and Schötz (2002) revealed that rutin is indispensable for the antidepressive activity of SJW extract in the FST. The re-examination of rutin in the FST showed, as expected, that rutin is active in concentrations comparable to that of imipramine. Furthermore, investigations regarding isorhamnetin, a possible metabolite of the *Hypericum* flavonoids, indicated a greater reduction of the immobility time than imipramine. These results lead to the assumption that *Hypericum* flavonoids, and especially their metabolites, take part in the extract's antidepressant activity, but to date no molecular mechanism has been identified. Recent *in vitro* receptor studies including various *Hypericum* flavonoids showed varying affinities to some receptors for flavonoids (Butterweck et al. 2001). However, these findings are of minor clinical relevance, because the substances tested, with the exception of miquelianin, are not available in either plasma or tissue. To elucidate the molecular mechanism of action of the flavonoids, further *in vitro* and *in vivo* studies, focused on the metabolites of the genuine flavonoids, are necessary.

These studies have attracted increased interest, since we demonstrated the ability of flavonoid metabolites to penetrate the blood-brain-barrier in a pilot animal study (Paulke et al. 2006). Moreover, the data of the present study, where a significant accumulation of isorhamnetin/tamarixetin in CNS tissue could be observed after multiple oral dosage of a SJW extract, makes it more likely that adequate CNS concentrations of flavonoids are achievable.

Accumulation of SJW flavonoids could perhaps partly explain the observed induction period of 2–4 weeks between the beginning of the oral administration and clinical effect of SJW extract *in vivo*.

In the current investigation we found a significant accumulation of the SJW flavonoids in the brain tissue of rats after multiple oral doses. Together with the *in vivo* studies these are strong indications for the participation of flavonoids in the antidepressive efficiency of the extract. Nevertheless the mode of action of SJW flavonoids including other neuromodulatory effects beside the antidepressant activity still remains unclear and requires further investigation. *In vitro* studies with flavonoid metabolites and clinical studies with flavonoid enriched extracts could provide additional data.

Based on our results we conclude that for medical effectiveness *Hypericum* preparations of a high pharmaceutical quality should contain sufficient amounts of both flavonoids and hyperforin in the SJW extract.

## 4. Experimental

### 4.1. Extracts and chemicals

SJW extract (WS® 5572) containing 1.35% isoquercitrin, 0.38% quercitrin, 3.26% rutin and 1.83% hyperoside all calculated as rutin, isoquercitrin and the flavon-free fodder were provided by Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe. HPLC-grade methanol, acetic acid (100%) and hydrochloric acid 30% (suprapure) was purchased from Merck, Darmstadt; HPLC-grade acetonitrile from Fisher scientific, Loughborough, UK, chloroacetic acid (99%), sodium chloroacetate (98%) tris buffer (99.9%), imipramine HCl and HPLC-grade ethanol from Sigma-Aldrich, Taufkirchen; *tert*-butylhydroquinone (HPLC) and isoquercitrin (HPLC) from Fluka, Steinheim; aluminium nitrate nonahydrate (p.a.) from Acros, Geel, Belgium; isorhamnetin (HPLC) and tamarixetin (HPLC) from Roth, Karlsruhe. Water was purified by a Milli-Q system (Millipore, Bedford, MA).

### 4.2. Animal studies

All animal studies were performed in accordance with the German Law of Animal Welfare (Tierschutzgesetz), accreditation numbers 35–9185.82/740/97, and 35–9185.82/714/97, Regierungspräsidium Karlsruhe). Male Sprague Dawley rats weighing between 200–300 g from Janvier, Le Genest St.Isle, France were used. Rats were maintained under standardised housing conditions (21–22 °C, 50–60% relative humidity, light from 6 : 00 to 18 : 00) for a period of at least 5 days prior to the start of the experiment.

### 4.3. Pharmacological studies

The animals received a standard maintenance diet and water *ad libitum*. All pure constituents and the extracts were suspended in 0.2% agarose gel. All test preparations were administered orally by gavage over a time period of 9 days at a volume of 10 ml/kg body weight (b.w.).

The duration of immobility in the forced swimming test (FST) was measured using the protocol described by Porsolt et al. (1979) with some modifications. After 7 days of drug administration, rats were trained to swim (15 min) in a glass cylinder (height 50 cm; diameter: 25 cm) containing 16–20 cm of water (25 °C). On day 9 (1 hour after the last drug administration) rats were tested for 5 min and the duration of immobility was measured.

However, drugs that increase motor activity may produce false positives in the FST; thus it was necessary to conduct the open field test in parallel to evaluate possible alterations of spontaneous motility and explorative activity of the extract or the vehicle.

The mobility of the animals was measured using an infrared photocell system (TSE, Germany). The animals were placed individually in a Plexiglas box (open-field) for 20 min. Then the animals were treated and the locomotor activity was measured for additional 2 h. Movements were quantified by the total time the rats were mobile, and the distance walked.

Statistics: Each value represents the mean ±S.D. of 8 animals/group. The mean values of the treatment groups were compared to those of the vehicle control group. For each experiment, the Bonferroni-Holm test procedure was applied to account for the multiplicity of tests and to achieve control of the multiple type I error rate ($^{*}\ p < 0.05$; $^{**}\ p < 0.01$).

### 4.4. Pharmacokinetic studies

The animals received a standard flavonoid free diet (Altromin, Germany) and water *ad libitum* 5 days before and during the study. Extract and isoquercitrin suspensions were made in 0.2% agarose gel and administered to the animals by gavage at a final volume of 10 ml/kg b.w. Blood and brain samples were taken at defined times after administration. The whole brain was carefully washed with ice-cold tris buffer solution (5 mM, pH 7.4), weighed and homogenized using a Potter-S, Braun (1 mL buffer/100 mg brain). Blood samples were collected directly into a Li-Heparin-Monovette (Fa. Sarstedt) containing 16 I.E. heparin/mL blood. The brain homogenates and the blood were stored for approximately 12 h at −20 °C before analysis.

#### 4.4.1. Pharmacokinetic study 1: (single dose)

The rats were divided into three groups: the placebo group containing 6 rats administered with 10 mL/kg of 0.2% agarose (vehicle), the isoquercitrin group containing 30 rats administered with 100 mg/kg isoquercitrin and the extract-group containing 30 rats administered with 1600 mg/kg

SJW extract. Samples were taken 4 h after administration for the placebo group and at 2, 4, 8, 24 and 48 h after administration for the isoquercitrin and extract groups. Each measuring point consists of 6 rats.

*4.4.2. Pharmacokinetic Study 2: (repeated doses)*

The rats were divided into two groups: The control group and the extract treated group. The control-group contained 6 rats, which were treated once per day with the vehicle (agarosegel 0.2%). After 8 days and 4 h after last treatment, the rats were dissected and samples were taken. The extract-group contained 18 rats treated once per day with 1600 mg/kg SJW extract. Measuring points of this group were every 4 h after the last feeding, on day 1, 5 and 8. Each measuring point contained 6 rats.

### *4.5. Analytical method*

For the sample preparation, HPLC apparatus and conditions, quantitation and standards see Paulke et al. (2006).

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