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Comparison of the synaptosomal uptake inhibition of serotonin by St John's wort products

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Abstract

Although the number of prescriptions for psychotropic drugs has decreased in recent years, prescriptions for antidepressants are still increasing (Fritze 2002). *Hypericum perforatum* (St John's wort) is the main psychotherapeutic herbal medicinal product used for treatment of mild-to-moderate depression. The lipophilic constituent hyperforin (2–5% of the extract) demonstrated, similarly to chemical antidepressants, a significant effect on the synaptosomal uptake inhibition of several neurotransmitters in in-vitro assays. In Germany, St John's wort products are distributed via two different markets: products that are pharmacy restricted are only allowed to be distributed in pharmacies; traditionally used products, which do not claim to have a curative character, are allowed to be sold in supermarkets. Depending on the market wherein a St John's wort product is offered, it needs to fulfill the legal requirements regarding pharmaceutical quality, safety and efficacy. Our goal was to compare the quality of St John's wort products distributed in pharmacies with that of those available from supermarkets. Therefore, the quantity of the pharmaceutical active ingredients (the phloroglucinol derivate hyperforin, the flavonoids rutin, hyperoside, isoquercitrin, quercitrin and the biflavonoid biapigenin) was determined by high-performance liquid chromatography (HPLC). The naphthodianthrones hypericines and pseudohypericines were quantified by differential pulse polarography (DPP). The efficacy of the products was investigated by measuring their activity to inhibit serotonin (5-HT) uptake in-vitro using a radio ligand uptake assay. It could be demonstrated that the products were different not only in the concentration of pharmaceutically relevant ingredients but also in showing individual IC50 values (concentration producing half-maximal inhibition) in the serotonin reuptake assay (IC50 values between 3.07 and 17.9 μg extract mL^{-1}). The results of our study confirm the assumption that the potency of St John's wort products in inhibiting the uptake of serotonin depends on the amount of hyperforin in their dosage forms. St John's wort products having greater hyperforin content and potency on synaptosomal serotonin uptake inhibition are restricted to be sold only in pharmacies.

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Introduction

St John's wort extract preparations are widely used in Germany to treat mild-to-moderate depression. Even though the mechanism of action is not yet completely clarified, pharmacological activity and chemical efficacy are documented by a large number of publications (Bhattacharya 1998; Chatterjee 1998; Kasper 2001; Lecrubier et al 2002). The good acceptance by patients, the major advantage of St John's wort over synthetic antidepressants, is mainly due to a low side-effect profile. St John's wort extract is a complex mixture of pharmaceutically important ingredients, including phloroglucinol derivates (hyperforin, adhyperforin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin), biflavonoids (biapigenin, amentoflavone) and naphthodianthrones (hypericin, pseudohypericin). From a pharmacological point of view, the question of which constituent is responsible for the pharmaceutical activity remains to be elucidated. The original assumption, that the inhibition of monoamine oxidase (MAO) by hypericin is responsible for the antidepressive activity of the extract, could not be confirmed in several independent studies (Cott 1997; Müller et al 1997). In synaptosomal preparations from rat or mice brain, hyperforin is a potent inhibitor of serotonin (5-HT), norepinephrine (noradrenaline), dopamine, gamma-aminobutyric acid (GABA) and L-glutamate uptake

with IC50 values (concentration producing half-maximal inhibition) of approximately 50–100 ng mL^{-1} (5-HT, noradrenaline, dopamine, GABA) and approximately 500 ng mL^{-1} (L-glutamate) (Müller et al 1998). Following a single dose (300 mg kg^{-1}) of St John's wort extract, which is effective in most animal behaviour studies, hyperforin reaches the plasma concentration in rats (~370 ng mL^{-1}) that is required to inhibit synaptosomal uptake of several neurotransmitters (Biber et al 1998). Clinical studies performed using St John's wort extract with different concentrations of hyperforin indicate that the antidepressive effect of St John's wort depends on its hyperforin content (Laakmann et al 1998; Berner et al 2002). The observed hyperforin plasma concentrations in man are 300 ng mL^{-1} after oral administration of 600 mg extract WS 5572 (Biber et al 1998). This concentration is very close to concentrations of hyperforin used in the in-vitro experiments to inhibit the synaptosomal uptake of 5-HT. The mechanism of action of hyperforin is unique, since it is the only antidepressant that inhibits uptake of 5-HT, noradrenaline and dopamine with similar potencies going along with a broader uptake-inhibiting profile, also inhibiting GABA and L-glutamate. This nonselective profile indicates that hyperforin acts in a different manner to classical antidepressive drugs. Indeed it could be demonstrated that hyperforin does not act as a competitive inhibitor at the transmitter binding sites of the proteins (Gobbi et al 1999; Singer et al 1999), as do chemical antidepressants, but shows a completely new mechanism of action. The driving force of all high-affinity neuronal neurotransmitter transport mechanisms mentioned is the sodium gradient between the high extracellular and low intracellular sodium concentrations. Interestingly, hyperforin reduces the sodium gradient by activation of a not-yet-characterized sodium conductivity mechanism, which then leads to inhibition of neurotransmitter uptake (Singer et al 1999; Müller 2003). It could be demonstrated that hyperforin interferes with the storage of monoamines in synaptic vesicles, rather than being a selective inhibitor of either synaptic membrane or vesicular monoamine transporters (Roz et al 2002). Another suggestion is that hyperforin may affect neurotransmitters uptake by dissipating the existing pH gradient generated by an efflux of inwardly pumped protons across the synaptic vesicle membrane (Roz & Rehavi 2003).

Most St John's wort brands on the German market are herbal medicinal products and there is a need to ensure constant quality and safety as well as efficacy, as pharmacy-restricted products approved on the German market, which are also reimbursed by the health insurance system. According to the German Drug Act, these herbal medicinal products are subject to the same regulatory requirements as chemical drugs. As an exception, the German law additionally allows distribution of herbal medicinal products in supermarkets when they do not claim to have a curative character. These products are not subject to strict requirements regarding quality, safety or efficacy as mentioned above. Their authorization goes back to their traditional application. In the case of St John's wort brands, these should have lower extract contents and are not labelled for the treatment of depression.

As an extension of our previous work where we investigated content uniformity and batch-to-batch reproducibility (Wurglics 2001a) followed by the biopharmaceutical characterization of the products (Westerhoff et al 2002), in this study we focused on the in-vitro pharmacology of St John's wort products from both markets, the pharmacy as well as the general market. We investigated the potency of St John's wort products to inhibit the synaptosomal uptake of serotonin. Four St John's wort products were purchased in a pharmacy and three products in a supermarket. The amount of pharmaceutically relevant ingredients was determined by HPLC and differential pulse polarography (DPP). Each product was also tested in-vitro for serotonin uptake inhibition. The target was to evaluate the differences in the pharmacological behaviour of St John's wort products when they differed in their amount of pharmaceutically relevant ingredients.

Materials and Methods

Materials

From the variety of St John's wort products on the German market seven products, as well as hyperforin sodium salt and the chemical antidepressants citalopram and clomipramine as positive controls, were tested in our study (Table 1). Four hypericum products were purchased in a pharmacy and three products were bought in a supermarket in Frankfurt/Main (Germany). The products were all analysed for their content of hyperforin, rutin, hyperoside, isoquercitrin, quercitrin, biapigenin and hypericines. Standard laboratory chemicals were obtained from Merck (Darmstadt, Germany), ethanol and methanol were from Roth (Karlsruhe, Germany), acetonitrile from Sigma Aldrich (Steinheim, Germany), HEPES from Gerba Biotechnik (Gaiber, Germany) and ^{3}H-serotonin creatinine sulfate (^{3}H-labelled 5-HT) from Perkin Elmer (Boston, USA). St John's wort extract WS 5572, hyperforin sodium salt, flavonoids and biapigenin standards were generously supplied by Dr Wilmar Schwabe GmbH & Co (Karlsruhe, Germany). Hypericin standard was obtained from Roth (Karlsruhe, Germany). Water was purified by a Milli-Q system (Millipore, Bedford, USA) and used for all aqueous procedures.

Sample analysis

To determine the composition of materials mentioned above, ten dosage forms from a single batch were weighed and milled together in an analytical mill. A tenth of the total mass was then quantitatively transferred into a 50-mL volumetric flask. The volume in the flask was brought to 40 mL with ethanol 60%. Extraction of the analytes was completed by subjecting the flask to 10 min of ultrasound at 30 °C. The volume was adjusted to 50 mL with ethanol 60% and samples of the resulting supernatant solution were removed by syringe and filtered through 0.45-μm filters (Rezist 30/0.45; Schleicher & Schuell, Germany). To determine the flavonoids and biapigenin a portion (200 μL) of

Table 1 St John's wort preparations.

	Product	Extraction solvent	Drug extraction ratio	mg extract/ dosage form	Recommended max. daily dosage
Pharmacy-restricted products	Neuroplant 1 × 1	Ethanol 60%	2.5–5:1	600	2 × 1
	Laif 900	Ethanol 80%	3–6:1	900	1 × 1
	Remotiv	No declaration	—	250	2 × 1
	Jarsin	Methanol 80%	3–6:1	300	3 × 1
Products for general sale	St Benedikt	Ethanol 60%	4–6:1	180	1 × 1
	Klosterfrau	Ethanol 70%	2.5–5:1	70–130	2 × 1
	Kneipp	Powdered dried herb	—	300	3 × 1

the filtered sample was transferred into an HPLC vial, containing 300 μL ethanol 60% and 500 μL of mobile phase. For the determination of hyperforin, the filtered supernatant was directly analysed by HPLC. The total flavonoid content, as well as the hyperforin concentration, was determined with a previously described HPLC method (Westerhoff et al 2002). For the quantification of biapigenin, the flavonoid HPLC method was optimized (Schulte-Löbbert et al 2003). The total hypericines content was determined with a published electrochemical method (Michelitsch et al 2000).

Synaptosomal uptake of serotonin

We used synaptosomal preparations to characterize the effects of the various St John's wort extract preparations using a previously described method (Singer et al 1999) with slight modifications. The frontal cortex of NMRI mice (Harlan Winkelmann, Borchen, Germany) were used. The tissue was homogenized in ice-cold sucrose solution (0.32 M) and diluted to 25 mL final volume. The nuclear fraction was eliminated by centrifugation at 7500 *g* for 10 min. Then the supernatant was centrifuged at 17 400 *g* for 20 min to obtain the crude synaptosomal pellet. This pellet was resuspended in approximately 11 mL ice-cold Krebs-HEPES buffer (composition (in mM unless stated otherwise):150 NaCl, 10 HEPES, 6.2 KCl, 1.2 Na_2HPO_4, 1.2 $MgSO_4$, 10 glucose, 10 μM Pargylin, 0.1% ascorbic acid; pH 7.4 at 37 °C) resulting in a tissue concentration of 30 mg mL^{-1} wet weight. The suspension was transferred to a 96-well microtitre plate immediately. The ethanolic supernatant solution obtained from each product after extraction was further diluted 1:10 with Krebs-HEPES buffer and used as test solution. Depending on the quantity of St John's wort extract in each product, the test solution was diluted with Krebs-HEPES buffer to obtain ten different extract concentrations in the range of approximately 0.04–1800 μg extract/mL of each St John's wort product. A sample of each dilution was added to the 96-well microtitre plate and incubated at 37 °C for 15 min in a shaking water bath. The ^{3}H-labelled 5-HT (2.9 nM) was added to the ice-cooled plate and the uptake experiment was started by incubation at 37 °C for 4 min. The plate was cooled on ice before being filtered through Whatman GF/B glass fibre filters and washed three times with ice-cold buffer solution with a Brandel cell harvester. The filters were placed in plastic vials and, after drying for 30 min at 60 °C, 4 mL Lumasafe scintillation fluid (Packard, Dreieich, Germany) was added to each vial. After shaking the vials for 2 h radioactivity was measured with a liquid scintillation analyser (1900 TR Counter, Canberra Packard, USA). Nonspecific uptake was determined in parallel experiments containing unlabelled 5-HT (1 mM).

Results

Each St John's wort product had its individual profile of pharmaceutical relevant ingredients. The detailed results show that the extracts exhibited quantitative differences in all quantified pharmaceutical ingredients. The highest variation, as expected, was found for the hyperforin values (Table 2), ranging from 0.02% (Remotiv) up to 3.9% (Neuroplant 1 × 1). Except for the total hypericines value, the quantity of pharmaceutically active ingredients of the product Kneipp was well below the average of the determined values of all other tested products. This might be due to the fact that Kneipp is made of St John's wort herbal powder instead of St John's wort extract. The percentage of flavonoids in the extracts was comparable among most products. The average content of flavonoids and biapigenin per dosage form in pharmacy-restricted products was much higher than in products from the supermarket. This correlated to the amount of St John's wort extract used in the products. The quantity of rutin in pharmacy-restricted St John's wort products was approximately 2–5 times higher than in products for general sale. The quantity of total hypericines in Neuroplant 1 × 1 and Laif 900 dosage forms was comparable. The average content of hypericines in the other tested products was approximately 4 times lower.

Summarizing the results of the quantitative analysis, it could be demonstrated that the quantity of pharmaceutically active ingredients in the products from the supermarket was lower than that in pharmacy-restricted products.

To assure the capability of the radio ligand uptake assay to discriminate between products with different concentrations of hyperforin, clomipramine, a classical antidepressive drug, citalopram, a selective serotonin reuptake

Table 2 Amount of hyperforin, flavonoids, biapigenin and hypericines in St John's wort preparations.

Preparation	Hyperforin	Rutin	Hyperoside/isoquercitrin	Quercitrin	Biapigenin	Hypericines
Neuroplant1 × 1	23.49 (3.9)	15.65 (2.61)	16.94 (2.82)	1.96 (0.33)	1.19 (0.2)	2.12 (0.35)
Laif 900	7.05 (0.78)	15.51 (1.72)	26.07 (2.9)	3.07 (0.34)	3.49 (0.38)	2.22 (0.24)
Remotiv	0.06 (0.02)	5.73 (2.29)	11.24 (4.5)	1.12 (0.45)	0.33 (0.13)	0.58 (0.23)
Jarsin 300	4.96 (1.65)	7.48 (2.49)	12.61 (4.2)	0.77 (0.26)	0.89 (0.3)	0.72 (0.24)
St Benedikt	0.56 (0.31)	3.72 (2.07)	4.96 (2.76)	0.59 (0.38)	0.16 (0.09)	0.4 (0.22)
Klosterfrau	1.30 (1)	4.27 (3.28)	5.09 (3.91)	0.52 (0.4)	0.45 (0.35)	0.69 (0.53)
Kneipp*	0.10 (0.03)*	1.86 (0.62)*	2.52 (0.84)*	0.18 (0.06)*	0.12 (0.04)*	0.76 (0.25)*

Data is represented in mg/dosage form (% in St John's wort extract, *% in St John's wort powder). Data obtained from HPLC-analysis. Data for hypericin was obtained from DPP.

Table 3 IC50 values of different St John's wort products on the synaptosomal uptake of serotonin.

St John's wort product/drug	IC50 value	Hyperforin/ dosage form
Citalopram	0.292 ± 0.220 ng/mL	—
Clomipramine	0.683 ± 0.260 ng/mL	—
Hyperforin sodium salt	0.435 ± 0.160 μg/mL	—
Neuroplant 1 × 1	4.47 ± 1.33 μg/mL	23.49 mg
Jarsin 300	3.07 ± 1.02 μg/mL	4.96 mg
Laif 900	13.99 ± 4.48 μg/mL	7.05 mg
Remotiv	17.90 ± 8.47 μg/mL	0.06 mg
St Benedikt	17.37 ± 6.43 μg/mL	0.56 mg
Klosterfrau	11.64 ± 3.48 μg/mL	1.3 mg
Kneipp	—	0.1 mg

Data are means of 6 independent experiments, each done in triplicate. Half-maximal inhibitor concentrations (IC50 values) are obtained by log-probit analysis of inhibitory curves (8–10 different concentrations).

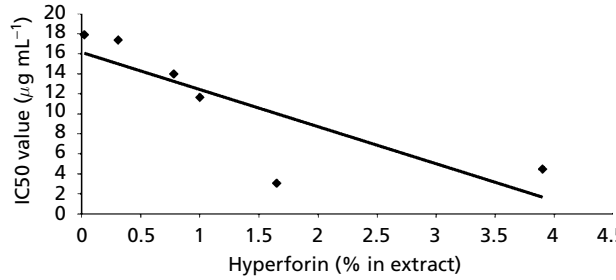

Figure 1 Two-side correlation analysis of the amount of hyperforin (%) and the experimentally determined IC50 values of different St John's wort products leads to $P = 0.0459/r^2 = 0.672$.

inhibitor (SSRI) and hyperforin sodium salt were tested in the assay as positive controls.

The investigation of the St John's wort products regarding their potency to inhibit the uptake of serotonin (Table 3) showed a clear dependency of the inhibition profile on the content of hyperforin. Correlation analyses (Figure 1) of the IC50 values and the percentage of hyperforin in the extract showed a significant relationship between serotonin reuptake inhibition and the determined content of hyperforin in each product ($P = 0.0459$). All other tested constituents failed to show a significant dependency, confirming that hyperforin is the major uptake-inhibiting constituent of St John's wort extract (Wonnemann et al 2001). Best results regarding uptake inhibition were obtained using Jarsin 300 and Neuroplant 1 × 1 in the radioligand uptake assay. The concentration of hyperforin in the extract of these products was over 1.5%, resulting in IC50 values of 3.07 $\mu g\,mL^{-1}$ and 4.47 $\mu g\,mL^{-1}$, respectively. St John's wort products with IC50 values over 17 $\mu g\,mL^{-1}$ were five times less potent than products with over 1.5% hyperforin in the extract. The product Kneipp showed no effect on uptake inhibition in the tested concentrations. This might be due to the fact that Kneipp is produced using hypericum powder, compared with the other products which contained hypericum dry extract in various concentrations.

Discussion

In Germany herbal medicinal products, such as St John's wort extract products, are subject to the same regulatory requirements as chemical drugs regarding their quality, safety and efficacy and are distributed in pharmacies only. Traditionally used St John's wort products are marketed in supermarkets where they are usually sold more cheaply. These brands do not need to fulfill the strictly legal requirements mentioned above and are not allowed to be labelled as antidepressants. It remains to be demonstrated whether these brands are at all useful based on the concentration of extract and ingredients.

In our study we focused on the number of ingredients that may contribute to the antidepressive activity of St John's wort extract, as well as their behaviour in the serotonin uptake assay. As previous studies demonstrated, we confirmed the variation in active ingredients in different St John's wort products (Wurglics et al 2001b). Pharmacy-restricted products had higher concentrations of

pharmaceutically relevant ingredients than products from the supermarket. The USP 26 monograph for powdered St John's wort extract requires a hyperforin concentration of at least 3%. According to our results only Neuroplant 1×1 fulfilled that requirement. The monograph also calls for a minimum total hypericin content of 0.2%, which was met by all tested products. Remarkable is the finding that the hyperforin concentrations of products from the supermarket were well below the values obtained from products which are pharmacy restricted. This finding correlates to the results of de los Reyes & Koda (2002). In a pharmacokinetic study in man it was reported that after administration of 300–1200 mg St John's wort extract WS 5572 (containing 5% hyperforin), plasma levels of approximately 150–437 ng mL^{-1} hyperforin could be measured after 3.5 h (Biber et al 1998). The recommended daily dosage of St John's wort extract is 600–900 mg. To reach pharmacologically active concentrations of hyperforin in plasma, oral administration of approximately 15–45 mg hyperforin is necessary. Comparing this range with the concentration of hyperforin in the dosage forms of the tested products, mainly Neuroplant 1×1 is dosed adequately when taken once a day. Jarsin 300 is recommended to be taken three times a day, giving a dose of approximately 15 mg hyperforin, which is still an adequate amount. Although Laif 900 contains high amounts of extract, the concentration of hyperforin in one dosage form is almost three times lower than in one dosage form of Neuroplant 1×1. Pharmacologically effective concentrations of hyperforin will not be reached by Laif 900 (7 mg hyperforin/dosage form), which is recommended to be taken once a day. To assure pharmacologically relevant plasma concentrations, products with hyperforin concentrations lower than 1% in the extract need to be taken more frequently than recommended by the manufacturer.

Earlier studies reported a weak inhibition of neuronal synaptosomal transmitter uptake by fractions containing oligomeric procyanidines (OPCs) (Wonnemann et al 2001). This might explain why St John's wort products containing almost no hyperforin show inhibition when tested for 5-HT uptake inhibition. Wonnemann et al (2001) evaluated five different St John's wort extracts with hyperforin levels under 0.3%. The corresponding IC50 values were between 15.5 and 119.8 μg mL^{-1}. These results can be verified by our survey. Extracts that contained less than 0.3% hyperforin had IC50 values of approximately 17 μg mL^{-1}. Since the OPCs belong to the hydrophilic fraction of St John's wort extract they certainly fail to cross the blood–brain barrier. Earlier experiments failed to detect any OPC activity in the Porsolt test (Butterweck et al 1997). Therefore their effect in the in-vitro assay can be neglected.

Summarizing the results, our data lead to the assumption that the German St John's wort market offers a variety of products that differ in their concentration of pharmaceutically relevant ingredients, as well as in their pharmacological behaviour in the in-vitro assay. For treatment of mild-to-moderate depression, a St John's wort product that guarantees a sufficient concentration of pharmaceutically active ingredients should be chosen to ensure the pharmacological efficacy of the product.

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