Pharmacological studies in an herbal drug combination of St. John's Wort (Hypericum perforatum) and passion flower (Passiflora incarnata): In vitro and in vivo evidence of synergy between Hypericum and Passiflora in antidepressant pharmacological models

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Abstract
Extracts of Hypericum, Passiflora and Valeriana are used for the treatment of mild depression and anxiety.
We were interested whether a combination of Hypericum and Passiflora exerts comparable effects to Hypericum alone. We used two well-established models for investigating extracts for their anti-depressant activity, namely the effects on synaptic uptake of serotonin and the forced-swimming-test. We show here for the first time, that Passiflora significantly enhances the pharmacological potency of Hypericum in both models.
Our data suggest that anti-depressive therapeutic effects of Hypericum are possible with lower doses, when it is combined with Passiflora, than with mono-preparations of Hypericum.

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1. Introduction
Extracts of Hypericum perforatum (St. John's Wort), Passiflora incarnata (passion flower) and Valeriana officinalis (valerian) are traditionally used for the treatment of mood and sleep disorders. For these three psychoactive herbal extracts, the extent of the evidence of their pharmacological characteristics varies greatly with most information being available for Hypericum extracts, which is one of the most sold phytomedicines in Europe.

In various models of depression, Hypericum extracts have been shown to act like conventional antidepressant drugs [1–3]. Hypericum exerts a variety of effects on central neurotransmitter systems [4,5]. The neurochemical mechanisms of the central actions of Hypericum are still debated but several components have antidepressant-like and anxiolytic-like effects in animals, or interact with neurotransmitter systems believed to be causally involved in depression, anxiety and in psychiatric illness generally. [6]. Many of the pharmacological activities appear to be attributable to the naphthodianthrone hypericin, the phloroglucinol derivative hyperforin and several flavonoids [6,7].

Passiflora is traditionally used in combination with other herbs as a mild sedative and there are limited published data relating to the pharmacology of the herbal extract alone [8]. After intra-peritoneal administration in mice, a reduction in motor activity, prolongation of sleep and anticonvulsant actions have been reported [9,10]. Sedative effects of passion flower extracts have been demonstrated in rodents [11]. Furthermore, Passiflora reveals anxiolytic effects [12–14]. The Passiflora constituent flavonoid, chrysin, has been shown to act as a partial agonist of benzodiazepine receptors and has
anxiolytic effects in mice but is not sedative nor a muscle relaxant [15,16].

One study, [17] using extracts of all three medicinal plants (Hypericum, Passiflora and Valerian), revealed that they activate GABA neurotransmission which is a pharmacological target of anxiolytic drugs as well as being a recognized target in the pharmacological treatment of depression. Recently, Elsas et al. demonstrated that elicit GABA currents in hippocampal neurons in vitro [12].

This study is part of a series of studies that aimed to establish the pharmacological characteristics of the proprietary combination of three special extracts of H. perforatum (Hypericum), P. incarnata (Passiflora) and V. officinalis (Valerian) in Neurapas® balance, which is registered in different European countries for the treatment of mild depression, anxiety and sleep disorders. The daily dose of the pharmacologically-active herbal extracts in the maximal recommended daily dose of Neurapas® balance are at the lower end of the recommended dose ranges that are presented in the respective monographs of the Kommission E of the German Drug Authorities [18–20]. The monographs describe the medicinal use of these herbs which differ from each other but together include the treatment of low mood (Hypericum), anxiety (Passiflora) and sleeping disorders (Valerian). These are common co-morbidities that may share a single pathophysiology [21] and this is the therapeutic rationale for the triple combination in Neurapas® balance. A neuropharmacological rationale for the combination of herbal extracts in Neurapas® balance has been proposed [22] and is based on the evidence that the combination is considered to act by activating serotonin and GABA neurotransmission, which are established targets in the pharmacological treatment of depression [23]. A randomized placebo-controlled clinical trial demonstrated that the drug has efficacy in treating mild depression showing a significant decrease in the Hamilton Depression Score of greater than 50% (own, unpublished data).

The aim of this study was to investigate possible synergistic effects of a Passiflora extract if it used in combination with a Hypericum extract using the forced swimming test [24,25] and in vitro uptake of neurotransmitters in rat synaptosomes [26], where extracts of Hypericum are reported to be active in both models ([7] own data).

2. Materials and methods

2.1. Herbal extracts

We used special dry extracts of H. perforatum (Hypericum), P. incarnata (Passiflora) and V. officinalis (Valerian) as combined in Neurapas® balance (PASCOE pharmazeutische Präparate GmbH). In addition, one other Hypericum extract with higher hyperforin content (2.7%) was included in the tests. The extracts were prepared by validated methods and the extraction solvents are all ethanol–water based.

The Hypericum extract is a dry extract of the aerial parts of H. perforatum with a ratio of the herbal drug to the herbal drug preparation of 4.6–6.5:1. Its hyperforin content is specified with not more than 1.5% (low hyperforin content). The extraction solvent is ethanol 38% (m/m). The hyperforin content of the tested Hypericum extract was 1.1% (low hyperforin extract). The second tested Hypericum extract had a hyperforin content of 2.7% (high hyperforin extract).

The Passiflora extract is a dry extract of the cut and dried aerial parts of P. incarnata containing flowers and leaves/or fruits. The extraction was performed in 60% (m/m) ethanol. The ratio of the herbal drug to the herbal drug preparation: 6.25–7.1:1 Extraction solvent: ethanol 60% (m/m).

As Harpagophyllum extract, used as a negative control here, is a dried aqueous-ethanol (40:60, vol:vol) extract (batch no. 7873/03) of the secondary storage roots of Harpagophyti procumbens (Hp) (PASCOE pharmazeutische Präparate GmbH). The content of the marker substance harpagoside in the native extract was calculated as 2.9% according to HPLC analysis.

The described dry extracts were used in the pharmacological studies and were dissolved in a measured small amount of dimethylsulfoxide (DMSO) and diluted in the application solution immediately prior to testing. All test doses contained the same quantity of DMSO as did all control test solutions. For animal studies, extracts were first dissolved in a small volume of 0.5% polyethyleneglycol and added an equivalent amount to the vehicle control solutions.

2.2. Serotonin re-uptake in rat synaptosomal preparations

Rat cerebral cortex was dissected freshly from Sprague–Dawley rats and immediately immersed in 10 volumes of ice-cold 0.32 M sucrose, buffered with 10 mM HEPES, pH 7.4 and homogenised with a Potter–Elvehjem homogenizer. Following centrifugation at 900 × g for 10 min at 4 °C the supernatant was collected and centrifuged at 10,000 × g at 4 °C. The pellet from this centrifugation was then kept in 0.32 M sucrose, buffered with 10 mM HEPES, pH 7.4 on ice prior to testing. For testing, the pellet was re-suspended in Famebo buffer (121 mM NaCl, 1.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 25 mM NaHCO3, 1.2 mM KH2PO4, 11 mM glucose, 0.57 mM ascorbic acid, 0.03 mM EDTA and 50 μM paraglyne) with a pH of 7.4.

Assays were carried in 180 μl Famebo buffer and each drug was tested at different final concentrations by addition of 10 μl of the diluted test substance, followed by 50 μl of the synaptosomal pellet suspension in 96-well filtration plates (Millipore Multiscreen) pre-wetted with Famebo buffer. The effect of the selective serotonin-reuptake inhibitor, Fluvoxamine® (10 μM), was tested in order to assess the potency of the test system (positive control). Following 10 min incubation, the tritiated serotonin ligand was added to a concentration of 2 nM and the incubation proceeded for 10 min. The incubation samples were then filtered with additional Famebo buffer. The synaptosomes with the internalized radiolabel remained on the filters which were then counted in a liquid scintillation counter with 50% additional Famebo buffer. The synaptosomal pellet suspension in 96-well filter plates was tested at different concentrations of test solution from 8 replicates. The specific uptake is defined astotal uptake minus uptake in the presence of 10 μM fluvoxamine. The EC50 values were calculated using the following formula:

\[
\text{specific uptake} = 1 - \left( \frac{I_{\text{max}}}{10^\text{[conc]}} \right) \div \left( 10^{-\text{EC50} / C3} + 10^{\text{[conc]}} \right)
\]

[conc] is the concentration of the test substance or dry extract.
3. Animals

All animal experiments were carried out according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals, and approved by the Institutional Animal Use and care committee at the University of Florida.

3.1. Forced swimming test

The forced swim test (FST) is based on the observation that rats when forced to swim in a restricted space from which they cannot escape will cease apparent attempts to escape and become immobile apart from small movements necessary to keep their heads above the water [24]. This characteristic immobile posture reflects a state of despair in the rat; it is assumed that the animals have “given up hope of escaping”. Immobility is reduced by a variety of antidepressant drugs including tricyclics, monoamine-oxidase-inhibitors (MAOs) and selective serotonin reuptake inhibitors. There is a significant correlation between clinical potency and potency of antidepressants in the forced swimming test that is unique to this model. In order to establish the specificity of the test data, a parallel testing in the open field model is required to exclude possible effects on mobility in general.

The Hypericum extract (low hyperforin content) was administered alone or in a combination with the Passiflora extract (Hypericum:Passiflora, 2:1 (m:m)). Different groups of rats (n = 10 animals per group) were treated with a single dose of the Hypericum extract or of the combination. Different doses of each were tested and administered by gavage three times (24, 5 and 1 h) prior to testing. The tests were performed as previously described [27]. Two sets of experiments were run each with a vehicle-treated and active control groups.

3.2. Open Field test

Open Field test was performed as previously described [27] in male rats (n = 8 animals per group) following the same pretreatment regime and with the same experimental groups as in the FST. This test is essential to exclude false positive results in the FST by a non-specific motor stimulation.

3.3. Statistical analysis

The forced swim test data was analyzed using Tukey's Multiple Comparison Test.

4. Results and discussion

We investigated the effects of Hypericum extract alone or in combination with Passiflora to characterize possible synergistic effects on well established in vitro and in vivo models used to identify anti-depressant effects of pharmacological compounds and herbal extracts.

Depressive disorders are commonly attributed to an imbalance in serotonergic neurotransmission. One approach to bring this imbalance back to balance consists in inhibiting the reuptake of serotonin into the neurons, thus increasing its extracellular levels. Serotonin transporter inhibitors have been shown to improve the clinical picture of depression. Among them are chemical entities like tricyclic antidepressants or herbal extracts as demonstrated here and by many others [28].

In the first of these experiments, the effect of pure tannins on serotonin uptake was tested in order to exclude that their presence have possible non-specific effects on the synaptic uptake of serotonin. This group of substances is commonly present in herbal extracts and is known to cause protein denaturation and may disturb in vitro biological experiments. The tannin mixture inhibited serotonin uptake and the IC50 value was 39.3 μg/ml as calculated using the maximal inhibitory effect of the mixture, which was only 61.6% of the control value. Thus, tannins were unable to reduce synaptosomal serotonin uptake completely and had effects only at concentrations greater than 50 μg/ml (see Fig. 1), which is far in excess of the levels of tannins present in the herbal extracts tested here. The almost 90% inhibitory effect of the Fluvoxamine® (the positive control) confirmed the potency of the synaptosomal system. An extract of Harpagophyllum was used as a negative control to avoid false positive effects in the assay due to the general use of extract preparations dissolved in DMSO. The traditional use of Devils claw extract is not linked to anxiety and depression and is not expected to exert a specific effect on 5-HT re-uptake. As shown in Fig. 1, right column, the extract of Harpagophyllum did not affect 5-HT re-uptake. In contrast, Hypericum (500 μg/ml) inhibited serotonin uptake by greater than 80% and so confirmed previous published finding [25]. These results indicate that the effect of Hypericum is a pharmacological effect and not a non-specific effect of any general herbal constituent.

In further experiments using this model, the effects of Hypericum combined with Passiflora on the inhibition of serotonin uptake were studied. Synergistic effects of this combination had been previously observed in rat brain slices (unpublished results). Different batches of the same proprietary extract were tested for their efficacy in the synaptosomal model. These different batches differed in their content of hyperforin, and were tested separately in the presence of increasing concentrations of the Passiflora extract. The results of these experiments are summarized in Table 1 and the dose response curves for Hypericum and Passiflora and the combination of both (with the low hyperforin extract) are shown in Fig. 2. As has been reported by others (for review see: [27–29]), these results demonstrate that the inhibitory potency of Hypericum extracts in this in vitro pharmacological model is related to the concentration of hyperforin present in the extract. The presence of the Passiflora extract to the Hypericum extract leads to a multiple increase of the potency of a low-hyperforin-containing Hypericum extract (Fig. 2); the IC50 value for inhibition of serotonin uptake was 88.2 μg/ml for the low hyperforin-containing Hypericum extract alone compared to a value of 14.0 μg/ml in the presence of the Passiflora extract (50 μg/ml) (Table 1).

Passiflora extract alone exhibited little potency in this model but the data reveal that the Passiflora extract exerts a novel synergistic effect on the inhibitory potency of Hypericum in this pharmacological model. The Passiflora extract exerted a dose-dependent shift of the Hypericum dose-response curve of both low- and high-hyperforin containing Hypericum extracts. The effect of the Passiflora extract was
more pronounced for the low-hyperforin extract with the effect that the difference in potency of the low- and high-hyperforin containing Hypericum extracts was eliminated. These findings are very significant with regard to the safe medicinal use of Hypericum-containing preparations. Besides being considered to be an important active ingredient of Hypericum, hyperforin is responsible for the well-documented and well-publicized problem of drug interactions that is associated with Hypericum [30]. The Hypericum extract in Neurapas® balance contains little hyperforin, a Hypericum constituent which is highly unstable and readily degrades [31]. These studies and our data clearly demonstrated that, although the potency of the inhibitory effect of Hypericum extract on serotonin-uptake increases with increasing hyperforin content, the presence of the Passiflora can compensate for the low hyperforin-content of the Hypericum extract used here. This implies that preparations that contain Passiflora in combination with a low-dose of a low-hyperforin containing Hypericum extract can be as effective in treating mood disorders as mono-preparations of Hypericum with a high hyperforin-content.

To confirm the observed synergy between Passiflora and Hypericum in vivo and to prove the hypothesis that a combination Passiflora and Hypericum using low doses of Hypericum is at least as potent as high doses of Hypericum alone, we used a well-established and validated animal model for screening of antidepressant activity of drugs, the rat forced swimming test [24]. A significant correlation exists between clinical efficacy and potency of antidepressants in reducing the induced immobility that is the basis of this pharmacological model. To proof the effects in the synaptosomal assay, we used Hypericum alone and in combination with Passiflora. The extract of Passiflora was not used alone, since it already failed to show significant effects of 5-HT re-uptake in the in vitro model.

The results of the forced swimming test experiments are shown in Fig. 3. The Hypericum extract alone exerted a significant inhibitory effect on the immobility of the rats at the two test concentrations (180 and 360 mg/kg), which were comparable to the effect of the positive control, Imipramine® (30 mg/kg). However, the most potent inhibitory effects in this series of experiments were seen with the combination of Hypericum and Passiflora. The combination at a dose of 135 mg/kg, and which contained just 90 mg/kg of the Hypericum extract (and 45 mg/kg passiflora extract), exerted the largest inhibitory effect that was greater than that of 360 mg/kg Hypericum alone or that of 30 mg/kg Imipramine®. Higher doses of the combination of Hypericum and Passiflora were, however, less effective and exerted no significant effect. Thus, the combination of Hypericum with Passiflora exhibits a U-shaped dose–response curve in this test model. This is a well-known phenomenon for the forced swimming test model and is also observed with chemical antidepressants [32]. All test substances and mixtures were without effects in the open-field mobility test which excludes possible direct effects on motor function (not shown). Swimming and climbing was not determined in this set up [24].

We show here for the first time that Passiflora synergistically enhances the anti-depressive effects of Hypericum on

![Fig. 1. Effects of tannins on the uptake of radiolabelled serotonin by rat synaptosomes (open bars) compared to single concentrations of Fluvoxamine (positive control, black bar, 10 μM), the hypericum extract (grey bar, 500 μg/ml) and an extract of Harpagophytum procumbens (striped bar, 500 μg/ml). The mean uptake of radiolabelled serotonin is expressed as a percentage of the mean amount taken up by untreated synaptosomes.](image)

<table>
<thead>
<tr>
<th>Test extracts</th>
<th>Hyperforin content</th>
<th>Mean IC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypericum</td>
<td>1.1%</td>
<td>88.2</td>
</tr>
<tr>
<td>Hypericum</td>
<td>2.7%</td>
<td>13.0</td>
</tr>
<tr>
<td>Hypericum + Passiflora 8 μg/ml</td>
<td>1.1%</td>
<td>23.4</td>
</tr>
<tr>
<td>Hypericum + Passiflora 8 μg/ml</td>
<td>2.7%</td>
<td>13.9</td>
</tr>
<tr>
<td>Hypericum + Passiflora 50 μg/ml</td>
<td>1.1%</td>
<td>14.0</td>
</tr>
<tr>
<td>Hypericum + Passiflora 50 μg/ml</td>
<td>2.7%</td>
<td>9.7</td>
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serotonin up-take in vitro and in the forced swim test in vivo. Passiflora extracts have been shown to reveal anxiolytic effects in models of anxiety [33–35] possibly mediated by the GABAergic system [36,37] as also shown for the triple combination of Hypericum, Passiflora and valerian in a synergistic manner [17]. So far, no reports are available of the effects of Passiflora on the serotonergic system and as anti-depressive herbal phytomedicine, whereas Hypericum is a well established anti-depressive drug (for review see [38]) in part due to the inhibition of serotonin re-uptake (for review see [29]) as also confirmed by the data shown here.

The synergistic effects of Passiflora and Hypericum on serotonin re-uptake might be explained by different binding sites on the transporter molecule affected by the extracts,
which would result in an allosteric modulation of the transporter. A similar effect is known e.g. for the binding of the two antidepressants escitalopram and R-citalopram to the serotonin transporter [39]. Modulations of the serotonin transporter activity might also result from altered phosphorylation states of the transporter molecule due to inhibition of protein kinases or phosphatases by the herbal extracts or altered protein–protein interactions caused by conformational changes after binding [40].

Further studies will reveal the mechanisms by which Passiflora synergistically enhances the effects of Hypericum especially on the serotonergic system. The demonstration of this possible synergy in two separate tests for antidepressant pharmacology, one in vitro and the other one in vivo, strongly strengthens the significance of the finding which has important implications for the medicinal use of these two herbal drugs. Additionally, the data imply that the specific low-dose combinations of Passiflora and Hypericum are an alternative to high-dose Hypericum mono-preparations.

Taken together, our data prove that the anti-depressive therapeutic effects of Hypericum are possible with lower doses, when it is combined with Passiflora as in Neurapas® balance, than with mono-preparations of Hypericum. This might be due to different mechanisms by which both extracts act on the neurotransmitter system.

5. Conflict of interest statement

Financial support for the analysis was provided by PASCOE pharmazeutische Präparate GmbH, the manufacturer of Neurapas® balance. The sponsor had no influence on the conduct of the analysis. T. Kammler and G. Weiss are employees of PASCOE pharmazeutische Präparate GmbH. B. L. Fiebich and R. Knörle have received funding from PASCOE pharmazeutische Präparate GmbH.

6. Submission declaration

The article has not been published previously and is approved by all authors. If accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

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References


