

Ex vivo/in vitro absorption of STW 5 (Iberogast[®]) and its extract components

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Abstract

To correlate the pharmacological effects of the fixed herbal combination STW 5 (Iberogast[®]) containing nine extract components with its confirmed clinical efficacy, ex vivo/in vitro absorption tests were performed. For the investigation, the everted gut sac technique and, in a pilot study, the Caco-2-cell model were used. The absorption rate of the extracts was determined by measuring characteristic marker substances of each of the individual extracts using HPLC or GC techniques. The results allow us to conclude that the investigated substances from STW 5 possess a good bioavailability, which is in accordance with the rapid onset of the therapeutic efficacy and explains its known pharmacological effects and clinical efficacy in terms of multiple drug action and multi-target therapy, respectively. © 2006 Elsevier GmbH. All rights reserved.

Keywords: Ex vivo/in vitro absorption studies; Everted gut sac; Caco-2; STW 5 (Iberogast[®]); *Iberis amara*; *Matricaria recutita*; *Carum carvi*; *Silybum marianum*; *Melissa officinalis*; *Mentha piperita*; *Chelidonium majus*; *Glycyrrhiza glabra*; Glucoiberin; Cucurbitacine E; Chelidonic acid

Introduction

The study of the absorption kinetic of plant extracts and in particular of their fixed combinations is often complicated, as they contain a large number of phytochemical compounds, of which the serum concentrations after oral administration do not reach the limit of detection. Nonetheless pharmacokinetic data are a

requirement for the evaluation of pharmacological effects and clinical efficacy. For proof of absorption they are also demanded by drug regulatory agencies in toxicological studies.

In the present study, the model of the everted gut sac of the rat (Wilson and Wiseman, 1954) was adapted to enable the investigation of the absorption of plant extracts and their fixed combination STW 5, which is used in the therapy of gastro-intestinal diseases. Pilot in vitro measurements concerning the absorption of glucoiberin and cucurbitacine E were also conducted in the Caco-2 model.

STW 5 is a fixed combination of hydroethanolic herbal extracts from bitter candy tuft (*Iberis amara totalis*; STW 6), peppermint leaves (*Menthae piperitae*

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folium; STW 5 K-II), chamomile flower (*Matricariae flos*; STW 5 K-III), liquorice root (*Liquiritiae radix*; STW 5 K-IV), angelica root (*Angelicae radix*; STW 5 K-V), caraway fruit (*Carvi fructus*; STW 5 K-VI), milk thistle fruit (*Silybi mariani fructus*; STW 5 K-VII), lemon balm leaves (*Melissae folium*; STW 5 K-VIII), and greater celandine herbs (*Chelidonii herba*; STW 5 K-IX) each of which is reported to have multiple pharmacological properties relevant in gastrointestinal pathophysiology (Wegener et al., 2006).

Materials and methods

Plant extracts studied

- Fresh plant extract
(1:2, extraction medium ethanol 50%) for
I. amara totalis (Bitter candytuft whole plant).
- Drug extracts
(1:3, extraction medium ethanol 30%) for
Matricariae flos (Chamomile flower)
Carvi fructus (Caraway fruit)
Silybi mariani fructus (Milk thistle fruit)
Melissae folium (Balm leaves)
Menthae piperitae folium (Peppermint leaves)
Chelidonii herba (Celandine herb)
Liquiritiae radix (Liquorice root)
- Fixed combination product from these extracts
STW 5 (Iberogast[®]), including also *A. radix* extract

The extracts were devoid of ethanol by freeze-drying and reconstituted for the studies by dissolving in the incubation medium. For determination of the concentration dependency of the absorption in the everted intestinal sac models 4–6 different concentrations of the extracts corresponding to 1–100 μl of the original extract/ml were prepared by dilution with the incubation medium.

Ex vivo absorption model – everted intestinal sac

- Male adult SD-rats (400–550 g body weight) were used. Experiments were conducted according to local legal preconditions.
- Everted intestinal sacs were prepared according to Wiseman (1961, modified), by quickly removing the small intestine from rats killed under CO₂-anesthesia and transferring it to the incubation solution saturated with carbogen.
- The jejunum was then cleansed, gently everted and divided to proximal, middle and distal segments, which were assigned equally to the test substances and concentrations for avoiding any influence of putative segmental differences. Sacs of 3–5 cm in

length were prepared, filled with incubation solution (100 $\mu\text{l}/\text{cm}$) and ligated on both ends. Each sac was then placed in an Erlenmeyer flask (100 ml) containing 30 ml of a solution of extract in incubation medium. At the end of the incubation time the sacs were removed, washed 3 times in tyrode solution, gently blotted dry, cut open and the serosal fluid drained into small tubes. Each sac was weighed before and after draining the serosal solution and the volume of the solution was calculated. The length of the everted gut sac in relaxed state was then measured.

- Incubation solutions were tyrode solution (or tissue culture solution TC 199 (Barthe et al., 1998; Cornaire et al., 2000), preliminary experiments only), at 37 °C, permanently aerated with carbogen.
- Concentrations of extracts tested were 1, 2, 5, 10, 20, 50, 100 $\mu\text{l}/\text{ml}$ for *Liquiritiae radix*, 10, 25, 50, 100 $\mu\text{l}/\text{ml}$ for *Matricariae flos* and *Carvi fructus*, 25, 50, 75, 100 $\mu\text{l}/\text{ml}$ for *I. amara totalis* and STW 5, and 5, 10, 50, 100 $\mu\text{l}/\text{ml}$ for the other extracts.
- Incubation time was 30 or 90 min. For each extract and extract concentrations 4–6 tests were performed ($n = 4–6$).
- Concentrations in the incubation medium were calculated as mean of concentrations pre and post incubation. Transport rates for the respective marker substances were calculated as ng/cm intestine from serosal concentrations and volumes and are given as means with standard errors (σ). Their relation to the concentrations in the incubation medium was calculated by correlation analysis.
- As indicators of mucosal damage, the liberation of lactate dehydrogenase (LDH), and alkaline phosphatase (AP) was determined for each of the plant extract solutions and, as control, for the incubation medium in preliminary experiments (Barthe et al., 1998).
- The protein content of the intestine was determined after alkaline hydrolysis of the sacs (Lowry et al., 1951).

In vitro absorption model – pilot study in Caco-2 cells

- Caco-2 cells were obtained from the American Type Culture Collection (ATCC). For absorption studies a 24 mm Transwell diffusion system with polycarbonate membranes (pore size 0.4 μm , membrane area 4.7 cm^2 , volume of apical (donor) compartment 1.5 cm, basal (acceptor) compartment 2.5 ml) was used (Artursson et al., 1996, 2001; Braun et al., 2000). Incubation time in absorption tests was 5 h. Every hour samples were taken from the basolateral side and the volume withdrawn was replaced with fresh medium, which was corrected for further calculations.

- Integrity of the cell layers was tested via light microscopy, measurement of the transepithelial resistance (TEER) and the apparent permeability coefficient (P_{app} , ^3H -mannitol-test). Only experiments with a TEER of above $300 \Omega \text{cm}^2$ at the end of the incubation time and P_{app} between 1×10^{-5} and 1×10^{-6} were evaluated (Artursson et al., 1996; Braun et al., 2000).
- The compounds studied were used as isolated chemical compounds dissolved in incubation medium or as extract attempting to achieve concentrations sufficiently above the limit of detection in the basal compartment.
- Lacking cytotoxicity of the test substance concentrations was determined in Caco-2 cells, using the methylthiazolyltetrazoliumbromide (MTT) method (Jager et al., 2002).

Analysis of characteristic phytochemical components

The absorption of the extracts was determined by measuring marker substances characteristic for each of the extracts studied, in each incubation solution before the start and at the end of the incubation time and in the serosal solution at the end of the incubation time.

The following marker substances were measured:

I. amara totalis: glucoiberin, cucurbitacines E and I (HPLC⁽¹⁾)

Matricariae flos: bisabololoxide A (GC⁽²⁾)

Carvi frucus: carvone (GC⁽²⁾)

Silybi mariani fructus: silibinin A and B (HPLC⁽²⁾)

Melissae folium: rosmarinic acid (HPLC⁽²⁾)

Menthae piperitae folium: menthol (GC⁽²⁾)

Chelidonii herba: chelidonic acid (HPLC⁽³⁾ (4))

Liquiritiae radix: glycyrrhizic acid (HPLC⁽²⁾)

According to: (1) Reichling and Saller, 2006, modified; (2) DAB/PhEur., modified, (3) Gradient HPLC with acetonitrile and tetrabutylammonium hydrogen sulfate solution (0–60 min 2–9% acetonitril) over octadecylsilyl silicagel (5 μm , 250 \times 4.6 mm), (4) As newer pharmacological (Vahlensiek et al., 1995; Germann et al., 2006; Heinle et al., 2006), pharmacokinetic (Kosina et al., 2004) and toxicological (Adler et al., 2006) studies show, the alkaloids do not determine the properties of the extract, but rather the more hydrophilic components, for which chelidonic acid is a characteristic marker.

Results

Pilot experiments

Before starting the measurements in intestinal segments, extended and detailed preliminary experiments

were conducted for defining optimized experimental conditions. As parameters of the integrity of the mucosa, the influence of the extracts on the release of LDH and AP from the everted sacs into the incubation medium was tested over the maximal incubation time. None of the extracts, even at the highest concentrations tested, did induce any enhancement of the release rates. It can therefore be supposed that they have no detrimental influence on the functional integrity of the mucosa. The tissue culture medium TC 199 did not show obvious advantages over the simpler tyrode solution according to measurements of enzyme release and absorption rates. To keep the experimental conditions as simple as possible the tyrode solution was thus chosen for the main experiments.

The absorption rates, calculated as a function of length (cm) of the intestinal sac, were constant for even longer incubation times (120 min) than those used in the main experiment (30 or 90 min). As concentrations of volatile oils and rosmarinic acid in the organ bath showed a measurable decrease over time, the longer incubation time was chosen only where suitable and necessary to obtain serosal concentrations sufficient for analysis. Intestinal protein content was linearly correlated to the length of the intestine.

Absorption studies in intestinal segments

For all extracts and for STW 5 the intestinal absorption of marker substances was confirmed (Figs. 1 and 2). The calculation of absorption rates was possible for all or at least for the higher concentrations of the extracts in the incubation medium. Only the absorption rate of cucurbitacines E and I in *I. amara* extract over 90 min was below the limit of detection of about 50–100 ng/cm, though the concentrations in the organ bath did not change significantly over the incubation time. The linear correlation between the absorption rate and the concentration in the incubation medium fits the data well, as shown by the correlation coefficients R^2 (all > 0.9). Thus all concentrations tested lay below saturation levels.

Pilot experiments in Caco-2 cells

In the Caco-2 model, some pilot measurements were conducted with glucoiberin and cucurbitacine E from *I. amara* extract, using substance concentrations well below cytotoxic levels. The uptake rate of pure glucoiberin was 360 ng/cm²/1 h and 570 ng/cm²/5 h using an apical concentration of 350 $\mu\text{g}/\text{ml}$. This result was reproducible when glucoiberin was incubated together with STW 5, 7.0 $\mu\text{l}/\text{ml}$, to give the same concentration in the acceptor compartment. The uptake of cucurbitacine E was not quantifiable, as basal concentrations did not

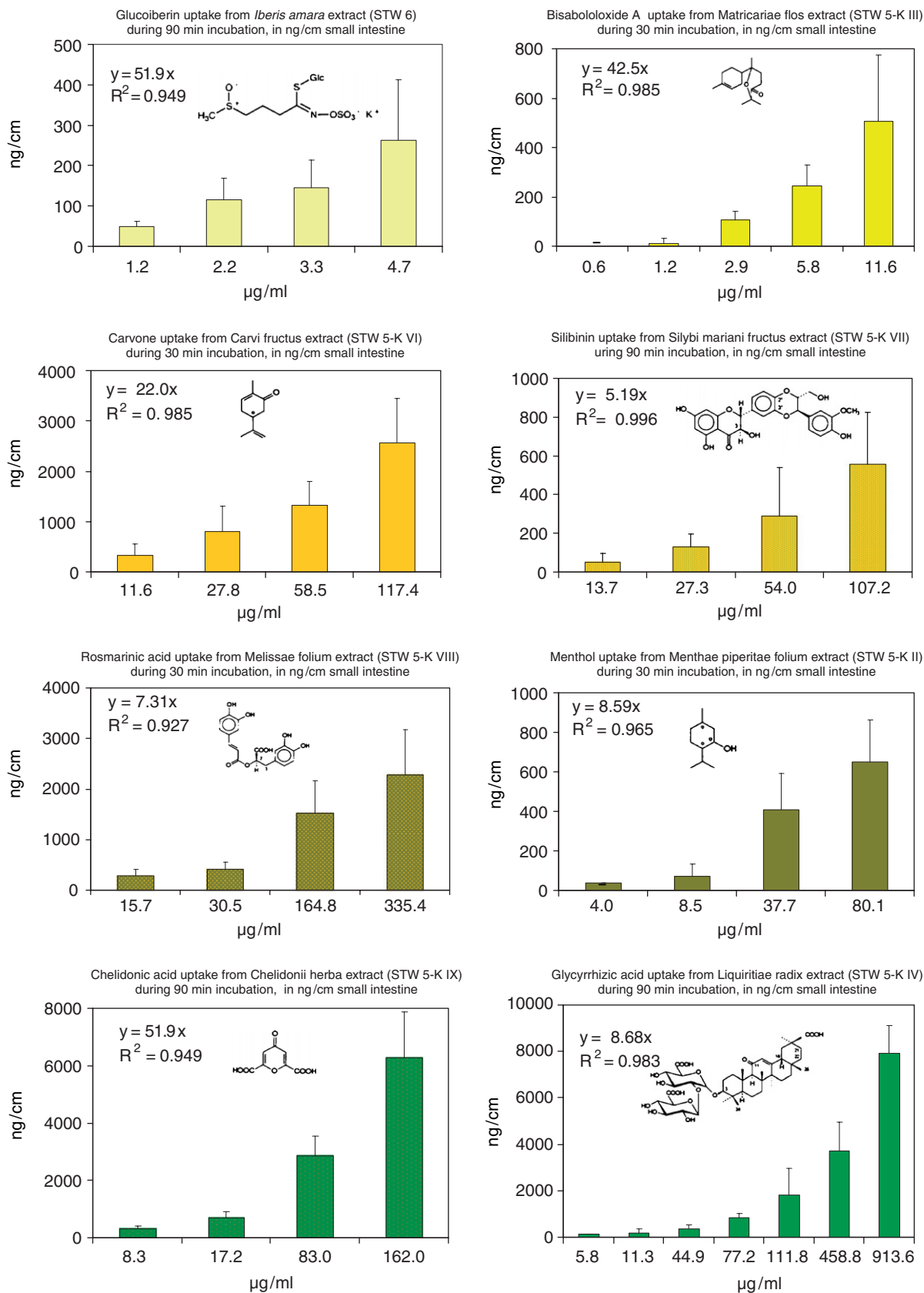


Fig. 1. Intestinal uptake rate (ng/cm) versus concentration of characteristic compounds from herbal extracts from STW 5 in the mucosal solution (µg/ml). Values are mean of 4–6 measurements \pm standard deviation. R^2 = correlation coefficient (linear correlation).

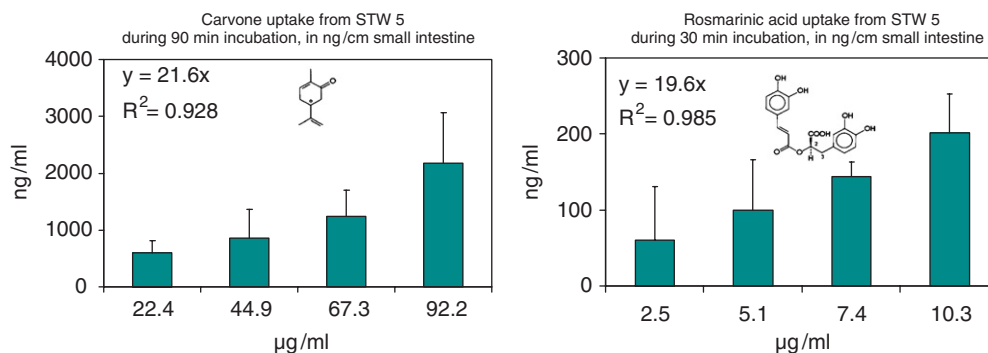


Fig. 2. Intestinal uptake rate (ng/cm) versus concentration of characteristic compounds from STW 5 in the mucosal solution (µg/ml). Values are mean of 4–6 measurements \pm standard deviation. R^2 = correlation coefficient (linear correlation).

reach the limit of detection while apical concentrations fell below that range over the 5 h incubation time.

Discussion

The model of the everted gut sac of the rat, developed by Wilson and Wiseman (Wilson and Wiseman, 1954; Wiseman, 1961), as well as the more recent Caco cell model, has its inevitable place in strategies for absorption screening in drug discovery and development (Barthe et al., 1998, 1999; Bohets et al., 2001). While the Caco-2 absorption model is a well defined cellular in vitro system based on a human colon adenocarcinoma cell line, the everted gut sac model is nearer to in vivo conditions and also, being simpler in handling and allowing a high sample throughput, without the methodological problems of in vivo perfusion models. In comparison to other models established to measure the uptake of characteristic phytochemical components from herbal extracts, it has as a special advantage the low volume of the acceptor compartment, related to the absorbing surface, so avoiding excessive dilution of absorbed substances. This is important due to their often low concentrations in the extracts, which rule out the quantification by the limit of detection of the analytical methods. Nevertheless it has been used for the study of phytochemical components from herbal extracts only recently, e.g. for the evaluation of extracts from *Stevia rebaudiana* (Koyama et al., 2003), *Perilla frutescens* (Baba et al., 2005) and *Astragalus* (Xu et al., 2006) or red wine (Dragoni et al., 2006).

The experimental conditions used in the studies presented here did not affect the integrity of the mucosa also in the highest extract concentrations tested. This has been proven in extended preliminary experiments, which showed no increase of the secretion of transaminases as parameter of cellular integrity, and no change of absorption rates over the incubation times used.

For *I. amara* extract, the measurements in the intestinal model show a significant, concentration

dependent uptake of glucoiberin. This was confirmed also in the Caco-2 cell model. For glucosinolates from another cruciferous plant, *Brassica oleracea italica*, human absorption studies pointed also to a rapid in vivo absorption (Kensler et al., 2005). Cucurbitacines E and I were not measurable in the serosal side, though concentrations at the mucosal side did not decrease significantly over the incubation time of 90 min and were more than 150 fold above limit of detection, pointing to a low absorption rate. As in the Caco-2 model concentrations in the donor compartment fell below the limit of detection within 5 h, a fast metabolic degradation may play a role, too. Accordingly, rapid metabolism of cucurbitacines E and I in S9-supernatants of rat livers was recently reported (Lorenz et al., 2005). This may contribute to the low toxicity of *I. amara* extract despite the possible toxicity of cucurbitacines, which has been reported for high doses of isolated cucurbitacines.

The data for the extracts from *Matricariae flos*, *Carvi fructus* and *Menthae herba* show a fast uptake of bisabololoxide A, carvone and menthol, respectively, linearly correlated to the mucosal concentration. Also for STW 5, the absorption of carvone was shown, with identical absorption rates as from the single extract (Fig. 2). Literature data on the in vivo uptake of menthol and carvone (Mascher et al., 2001) are in line with these results and point to glucuronidation as phase 2 reaction.

Silibinin was absorbed from the extract of *Silybi mariani fructus*, with absorption rates linearly correlated to the administered concentration. Human data confirm this (Savio et al., 1998) and indicate a metabolism by conjugation to glucuronide and sulphate (Mennicke, 1975; Büllens et al., 1975).

Dose-dependent absorption of rosmarinic acid is confirmed for the extract of *Melissae herba*, as well as for STW 5, where its concentration is 1/10. In both cases absorption rates were linearly dependent from the concentrations. In vitro and in vivo data from rat (Konishi et al., 2005) as well as from the Caco-2 model

(Konishi and Kobayashi, 2005) point to paracellular absorption for rosmarinic acid. Additionally, it can be assumed that rosmarinic acid is intestinally metabolized to *m*-coumaric acid and hydroxylated phenylpropionic acids, which are then absorbed more efficiently by the specific intestinal monocarboxylic acid transporter (MCT). Furthermore, human data with *P. frutescens* extract show an efficient absorption also of rosmarinic acid in vivo (Baba et al., 2004, 2005).

Proof of absorption of *Chelidonii herba* extract was done by the measurement of chelidonic acid, which is also absorbed linearly dependent from mucosal concentrations.

Glycyrrhizic acid uptake from the extract of *Liquiritiae radix* could, due to the high sensitivity of the analytical method, be shown over a very wide concentration range, showing linearity. This is in accordance with studies on the uptake of the aglycone of glycyrrhizic acid, glycyrrhetic acid, after oral administration in rats (Ishida et al., 1989a, b, 1990). Differing uptake rates from isolated glycyrrhizic acid and the extract were reported from Cantelli-Forti et al. (1997) in rats and in studies in humans from Terasawa et al. (1986). The uptake of glycyrrhizic acid to blood plasma has been shown after single as well as after repeated oral administration of STW 5 in toxicokinetic studies in the rat, rabbit and dog (unpublished results).

While not tested in this study, literature data also show the absorption of constituents of *Angelicae radix*, the furanocoumarins. After oral and cutaneous administration, they were well absorbed in humans (Teuscher and Lindequist, 1987).

The absorption data obtained for characteristic phytochemical components from the extracts contained in STW 5 are therefore in accordance with the literature. Due to the fact that only the unmetabolized compounds were detected by the analytical methods used, absorption rates are in tendency underestimated, as metabolites, including conjugates, are not included. Further studies are needed to cover also these metabolic compounds.

The linear form of absorption kinetics also in low concentration ranges could possibly explain in part the quick onset of the pharmacological action of STW 5. There were no apparent differences between the absorption from the extracts and from their combination STW 5, so allowing conclusions from extract data to STW 5. The linear form of absorption kinetics also in low concentration ranges could possibly explain in part the rapid onset of the pharmacological action of STW 5.

Conclusions

The absorption of characteristic phytochemical components from plant extracts contained in STW 5 (Iberogast®) has been confirmed using a uniform

method, the ex vivo/in vitro technique of the everted gut sac. The results are in excellent accordance with in vitro and in vivo literature data. They showed a linear dependency of the absorption rates from the concentration and allow the conclusion that a multitude of phytochemical components from STW 5 possess a good bioavailability, which can explain its known pharmacological effects and clinical efficacy in terms of a multi-drug and multi-target therapy (Wagner, 2005).

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