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In Vitro Activity of St. John's Wort Against Cytochrome P450 Isozymes and P-Glycoprotein

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Abstract

The inhibitory activity of 18 commercial St. John's wort products (*Hypericum perforatum* L. Hypericaceae) against human cytochrome P450 enzymes was assessed because recent studies have found that this herb can markedly affect disposition of concurrently used conventional drugs. For the most part, these studies have employed ethanolic extracts. However, many of the two dozen reported constituents or groups of compounds having pharmacological effects in *Hypericum* extracts have widely differing solubility characteristics and hence the interpretation of the results is problematic. Sequential extracts from hexane through to water demonstrated a strong effect of several lipophilic fractions on the cytochrome P450 3A4 mediated-metabolism of 7-benzyloxyresorufin (7-BR), suggesting that many different classes of compounds are active. In this study we sought to investigate 18 single-entity and blended products containing St. John's wort, including 7 caplets/tablets, 6 capsules, 4 teas and 1 tincture, for biomarker content and affect on cytochrome P450-mediated metabolism. Our results show that there is a wide variation in hyperforin, hypericin and pseudohypericin levels and that most standardized products did not meet specification. Furthermore, all aqueous extracts from the products tested exhibited a marked capacity to inhibit cytochrome P450-mediated metabolism. Four of the five extracts tested also inhibited P-glycoprotein activity. These findings support the notion that a wide range of therapeutic products used in conjunction with St. John's wort could lead to adverse side effects.

Keywords: St. John's wort, cytochrome P450, 3A4, P-glycoprotein, metabolism, inhibition, hypericin, hyperforin, drug interactions, natural health products

Introduction

St. John's wort (*Hypericum perforatum* L. Hypericaceae), a medicinal herb used since antiquity, has recently gained popularity as a herbal alternative for the treatment of mild to moderate depression. The plant is often used to treat a variety of conditions including depressed mood, anxiety, nervous unrest, excitability, neuralgia, fibrositis, sciatica, mood disturbances of menopause, fatigue, lack of drive, palpitations, exhaustion, headache, muscle pain, loss of appetite, insomnia, gastric indigestion, and topical treatment of injuries (Hahn, 1992; Jellin, 2000). In placebo controlled clinical trials, St. John's wort extracts, especially those enriched in hyperforin, reduced clinical scores for depression (Laakman et al., 1998).

Herbal products in general are now widely available and frequently used, yet they generally lack the stringent regulation of therapeutic products. As an increasing number of people include herbals in their diet, it is important that users and health care professionals be aware of any consequences and possible side effects involved with their use, particularly when used concomitantly with conventional therapeutic products. Current herbal products have a range of different

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information on their labels, some having no warnings at all, and others giving detailed cautions. The label warnings some of the products from this study included the following cautionary statements indicating that some manufacturers are attempting to ensure responsible use: "do not use the product during pregnancy and lactation or give it to young children, do not combine the product with prescription drugs or alcohol, and avoid sunlight due to the occurrence of increased photosensitivity of the skin". Some labels still note the medieval beliefs and uses such as "gaining a brighter outlook on life, lifting spirits and of the plant being a protector against evil" (Hahn, 1992).

There has been much literature recently examining the safety of St. John's wort (Beckman et al., 2000; Ernst, 2000; Gold et al., 2001; Greeson et al., 2001; Ingram et al., 2000; Miller, 1998; Nebel et al., 1999; Ness et al., 1999; Parker et al., 2001). Several reports have also examined potential drug interactions with St. John's wort (Breidenbach et al., 2000; Budzinski et al., 2000; Cheng, 2000; de Maat et al., 2001; Markowitz et al., 2000; Obach, 2000; Piscitelli et al., 2000, Roby et al., 2000; Ruschitzka et al., 2000). One noteworthy report dealt with heart transplant patients medicating with St. John's wort over a 3 week period of time, who started having acute rejection symptoms. The findings showed that the herb actually lowered the patient's plasma levels of the immunosuppressant cyclosporine (Ruschitzka et al., 2000). St. John's wort was also reported to lower the blood levels of the AIDS drug indinavir in healthy individuals (Piscitelli et al., 2000). Both of these therapeutic products are involved with cytochrome P450 3A metabolism. Other studies gave conflicting results. A small 14 day study with 8 healthy subjects found that St. John's wort (standardized to 0.3% hypericin) did not affect cabamazepine pharmacokinetics (Burstein et al., 2000). A shorter 4 day study with 7 healthy individuals taking St. John's wort reported that St. John's wort was unlikely to cause 3A4 and 2D6 inhibition (Markowitz et al., 2000). The reason for this lack of inhibition however, may be due to the small patient numbers and the short length of time involved as seen in the previous reports. Pharmacological effects have been attributed to at least two dozen constituents or groups of compounds present in *Hypericum* extracts (Moore et al., 2000; Wagner & Bladt, 1994). In addition to proanthrocyanidins like hypericin and pseudohypericin, the other major constituents are flavonoids such as quercetin, hyperoside, quercitrin, isoquercitrin, rutin, campherol, luteolin and I3-II8-biapigenin (about 2–4%) procyanidines; such as hyperforin (about 8%) phloroglucinols; and water-soluble components such as organic acids (chlorogenic), polysaccharides, tannins, and others. Hence, standardization to a single constituent may give insufficient information for comparing the capacity of a sample to affect drug interactions.

A major concern with natural health products is the variation in the quality of the product available. There is no regulation in this area and St. John's wort crops can often vary in their levels of biomarkers and other secondary metabolites (which may include other active ingredients) because of genetic and environmental factors (Büter et al., 1998) and seasonal variations (Southwell & Bourke, 2001). Also, consumer demand has resulted in manufacturing practices with differing quality levels and the use of other similar species with differing composition and no proven efficacy have been sold on occasion (Kurth & Spreemann, 1998). The general spectrophotometric determination method for standardization of naphthodianthrones is not as selective as HPLC is in identifying the related components and can be manipulated by colorant adulteration (Kurth & Spreemann, 1998). Variation in biomarker substances was also noted in broadleaved (370–580 ppm) and narrow leaved (1040–1630 ppm) varieties (Southwell & Campbell 1991). They reported that within a broadleaved sample, the main stem contained 40 ppm, side stem 120 ppm, bottom leaf 290 ppm, top leaf 380 ppm, capsules 730 ppm and flowers 2150 ppm.

Ethanolic extracts of two different brands of St. John's wort capsules along with one brand of tablets were shown to inhibit the *in vitro* activity of CYP 1A2, 2C9, 2C19, 2D6 and 3A4 isozymes (Obach, 2000; Roby et al., 2000). Durr et al. (2000) reported that intestinal P-glycoprotein/MDR1 (rats and humans), hepatic CYP3A2 (rat) and intestinal and hepatic CYP3A4 (human) was induced by extracts of St. John's wort. Ethanolic extracts from three capsule preparations of St. John's wort and a number of authentic substances present in the extracts were tested for activity with the pregnane X receptor (PXR) that regulates expression of cytochrome P450 3A4 (Moore et al., 2000). All three extracts and hyperforin, but not hypericin, were reported to be a potent PXR activator. Based on these findings the expected levels of hyperforin in a standardized product using a standard regimen of 3×300 mg should exceed the level required to activate PXR.

The study presented here is a confirmation of that of Roby et al. (2000), Obach (2000), and our previous tincture study (Budzinski et al., 2000). It expands these previous findings with a wider variety of single-entity and blended products (5 capsules, 8 caplets and tablets, and 4 teas) on the metabolism of marker substrates by selected human cytochrome P450 (CYP) isozymes (2C9, 2C19, 2D6, 3A4,). In addition we assayed the activity of each product on P-glycoprotein (P-gp) membrane drug transport using an ATPase assay. HPLC was used to determine the hyperforin, hypericin and pseudohypericin content in 22 samples from 18 products. Another important new tool is the use of the CYP polymorphism 2C9*2 along with 3A5 and 3A7 to examine the possibility that St. John's wort water extract could have similar inhibitory effects on these isoforms.

Materials and methods

Substrates and reference compounds

The St. John's wort single-entity and blended capsules, tablets and teas were obtained at local outlets. These were

Table 1. Test product label information on weight (listed and measured), hypericin or hyperforin percentage, suggested dose, other ingredients, sources and the physical appearance of the product.

NRP*	Stated Wt × Amount (avg. unit wt)	Suggested Dose	Other Ingredients	Sources (appearance of product)
8b,c	1.42 g tea bags		ingredients not listed	(small fibres of plant material)
93	300 mg tablets (753.3 mg) 0.3% hypericin	one 3 × daily (900 mg/day)	sucrose, lactose, talc, powdered cellulose, hydroxypropyl methylcellulose, polyethylene glycol, castor oil, magnesium stearate, polyvinylpyrrolidone, silicon dioxide, gelatin, titanium dioxide, carnauba wax	gently dried special extract from upper parts of flowers and leaves
94	150 mg capsules (total 497.6 mg; powder 420.2 mg) 0.2% hypericin	two 3 × daily (900 mg/day)	rice flour, capsule contains gelatin and purified water	(light grey powder)
95	450 mg capsules (total 641.5 mg; powder 522.5 mg) 0.3% hypericin	two 1 × daily (900 mg/day)	ethylcellulose, maltodextrin, silicon dioxide, capsule contains gelatin and purified water	powdered St. John's wort aerial parts extract (brown, powder/solid clumps)
96	200 mg tablets (427.2 mg) 0.3% hypericin	one 3 × daily (600 mg/day)	cellulose, croscarmellose sodium, magnesium stearate	powdered St. John's wort aerial parts extract
97	300 mg tablets (566.2 mg) minimum 3% hyperforin	one 3 × daily (900 mg/day)	microcrystalline cellulose, corn starch, croscarmellose sodium, hydroxypropyl methylcellulose, polyethylene glycol, magnesium stearate, silicon dioxide, ascorbic acid, iron oxide, titanium dioxide, talc, pharsil, vanillin	(brown/yellow tablet)
98	992.25 mg capsules (total 418 mg; powder 321.1 mg) 0.3% hypericin	one 3 × daily (2.98 g/day)	gelatin	leaves and flowers (brown powder)
99	300 mg capsules (total 647.6 mg; powder 540.1 mg) 0.3% hypericin	one 3 × daily (900 mg/day)	gelatin capsule, rice protein, vegetable grade magnesium stearate	(mixed light and dark particles)
100	500 mg tablets (505.6 mg) 0.3% hypericin	one-two 3 × daily (1.5–3.0 g/day)	microcrystalline cellulose, magnesium stearate	dried aerial parts of *Hypericum perforatum*
101	100 ml 200 mg	2–4 ml 3 × daily (400–800 mg/day)	45% alcohol	dried aerial parts of *H. perforatum* liquid extract 1 : 1

Table 1. Continued

NRP*	Stated Wt × Amount (avg. unit wt)	Suggested Dose	Other Ingredients	Sources (appearance of product)
102	945 mg capsules (total 523.7 mg; powder 403.5 mg) 0.3% hypericin	one 3 × daily (2.84 g/day)	gelatin, *Ginkgo biloba* leaf, Siberian ginseng root, ginger rhizome.	leaves and flowers, solid extract 1 : 4.5 (brown/yellow powder/fibres)
103	300 mg caplets (904.3 mg) 0.3% hypericin	one-two before sleep when required (300–600 mg)	*G. biloba*, lemon balm whole leaf, rosemary whole leaf, calcium phosphate, microcrystalline cellulose, modified cellulose gum, stearic acid, magnesium stearate, silica, water, cellulose, polyethylene glycol	full spectrum powdered extract 1 : 4
104	225 mg tablets (489.5 mg) 0.3% hypericin	two-four 3 × daily (1.35–2.7 g/day)	ingredients not listed	powdered extract of dried aerial parts of flower, stamen and leaf
105a,b,c	1.7 g tea bags		ingredients not listed	(small fibres of plant material)
118a,b	1.5 g tea bags 0.3% hypericin		lemon balm leaf, oatstraw herb, damiana leaf, lavender flower, bergamot herb, sage leaf, spearmint leaf, lemongrass leaf, licorice root, rose petal, stevia leaf	flowering tops and standardized extract (small fibres of plant material)
119b	850 mg tea bags		fennel seed, cinnamon bark, spearmint leaf, fenugreek seed, ginger root, cardamom seed, lavender flowers, natural licorice flavour, clove bud and black pepper	leaf and flower tops (small fibres of plant material)
128	300 mg tablets (850.0 mg) 0.3% hypericin	one 3 × daily (900 mg/day)	kelp, peppermint leaf, scullap, Siberian ginseng, wood betony, calcium phosphate, microcrystalline cellulose, vegetable stearin, croscarmellose sodium, silicon dioxide, magnesium stearate	powdered extract 1 : 5
129	300 mg capsules (total 622.3 mg; powder 526.0 mg) 0.3% hypericin (900 µg)	one 3 × daily (900 mg/day)	gelatin capsule, magnesium stearate, rice protein	(dark grey powder)

* NRP, Nutraceutical Research Programme accession number.

assigned Nutraceutical Research Programme (NRP) accession numbers and vouchers stored in the herbarium, University of Ottawa. 7-Benzyloxyresorufin (7BR) was purchased from Sigma Chemical Co, MO, USA. 7-Ethoxy-3-cyanocoumarin (CEC) was obtained from Molecular Probes, OR, USA. 7-Methoxy-4-trifluoromethylcoumarin (7-MFC) was obtained from Fluka Chemicals, Switzerland. 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) was obtained from GENTEST Corp., MA, USA. Hyperforin was obtained from Addipharma, Gmbh & Co., Hamburg. Hypericin was a generous gift from VIMRx Pharmaceuticals Ltd, Stamford, Conn. All other chemicals and solvents were of analytical grade.

Aqueous extractions

Stock solutions of the aqueous extraction samples of St. John's wort were prepared unless stated othewise at room temperature under reduced lighting conditions from the plant material within capsules, tea bags, and whole tablets. Each sample was mixed with deionized water in a 50 ml Falcon polypropylene tube, making either a 25 mg/ml solution (capsules and tablets) or a 100 mg/ml solution (teas). Each was then homogenized for 1 min to a consistent suspension using a Polytron. This solution was placed in 1.5 ml microfuge tubes and centrifuged for 18 min at 13,000 rpm. The stock solutions were diluted as required.

Infusion stock solutions were prepared by adding a single tea bag to 200 ml of deionized water at 100 °C in a 250 ml Erlenmeyer flask and placing it in a Series 25 Incubator Shaker (New Brunswick Scientific Co., INC.) at room temperature at 100 rpm for 15 min. The tea bags were gently pressed, then removed and discarded. The infusions (in triplicate) were combined and filtered using a Buchner funnel with Whatman 1 qualitative 70 mm filter papers to remove larger particulate matter. Samples were then filter sterilized using Nalgene 500 ml, 0.2 µ pore size nylon filter units and stored at −80 °C.

Sequential solvent extractions

The content of a St. John's wort capsule was mixed with hexane in a glass centrifuge tube to make a 25 mg/ml solution, which was then blended for 1 min using a Polytron. The homogenate was centrifuged for 15 min at 2500 rpm. After removing the hexane supernatant to a separate 5 ml glass tube, the remaining solid material was mixed with the same amount of hexane again, vortexed, sonicated and centrifuged for 15 min. This step was repeated until the hexane supernatant was clear and colourless. All hexane fractions were collected into the same 5 ml tube and evaporated to dryness *in vacuo*. The capsular material left in the original tube was extracted again with chloroform. As above, these steps were repeated until the supernatant was clear and the chloroform fractions were collected in a 5 ml glass tube and evaporated to dryness. The extraction steps were repeated with ethyl acetate and methanol. The residues left after evaporating the fractions from each solvent were resuspended in 1 ml of methanol to make a 100 mg/ml solution to be tested. Ethanol (55%) and water were the last two solvents used for this series of extractions. The procedure used for these solvents was the same as that used for the others, except that the fractions which were collected were not evaporated to dryness (due to the excessive length of time required). Instead, they were tested as is at 25 mg/ml. All extracts had appropriate controls.

Phytochemical content analysis

Twenty-two samples from 18 St. John's wort products were analyzed as follows: Products were ground in a Wiley mill and passed through a 40 mesh screen. Ten ml of 70% acetone (to release hypericin and pseudohypericin) was added to a first unit (content of one capsule, 0.2 g of ground tea, or one tablet) of each sample, as well as 10 ml of 99% ethanol (to release hyperforin) to a second unit, in individual centrifuge tubes. For tablet samples in ethanol, an additional 3 ml of distilled water and 3 ml of 70% acetone was added to the first extraction step to facilitate their dissolution. Tubes were inverted 3 times, sonicated for 10 min and centrifuged at 1000 × g for 5 min (tablets were left in solvent overnight; teas and capsules extracted immediately after the addition of the solvent). Supernatants were poured into separate amber glass bottles. The solid remaining sediments were extracted as described previously 3 more times with 10 ml of the corresponding solvent. Supernatants for each sample were combined and volumes in the bottles were adjusted to 40 ml with the solvent (except for ethanol samples, volumes were noted). A 1.5 ml aliquot of each sample was filtered through a 0.22 µm PTFE membrane and put into amber glass HPLC vials. Samples were analyzed by HPLC using a 3 µ LiChrospher 100-RP-18, 75 × 4.6 mm analytical cartridge and a 5 µ LiChrospher RP-18, 4.6 mm guard cartridge. The sample were run under the following system conditions: Temp = 45 °C; flow rate = 1.5 ml/min; solvents = 50 mM Na_2HPO_4 pH 7.1 with H_3PO_4 (A) and acetonitrile (B); linear gradient = 10–25% B in 5 min, hold 90% B 2.5 min, 90–10% in 2.5 min and equilibrate 7 min; detection = 587 nm (hypericin and pseudohypericin) and 290 nm (hyperforin). The amount of compounds present were detected in µg/ml. The HPLC was a Beckman system consisting of a Module 502 autosampler, a Module 126 solvent delivery system, a Module 168 photo diode array detector and version 8.10 system Gold software.

All samples were covered with aluminum foil or kept under reduced lighting conditions to prevent light-sensitive reactions.

Cytochrome P450 assay procedures

Aliquots (5 µl) of stock solutions from the extracts were screened for their ability to inhibit 2C9*1, 2C9*2, 2C19, 2D6, 3A4, 3A5 and 3A7 marker substrates using an *in vitro* fluorometric microtiter plate assay. The procedure was reported previously (Foster et al., 2001). Test-blank wells consisted of the corresponding extract and buffer solution. In addition, selected products were incubated as above with CYP3A4 and testosterone as the marker substrate to determine the relative amount of 6-β-hydroxytestosterone formed. The reactions were stopped with an equal volume of methanol. Samples were centrifuged and filtered prior to analysis by HPLC. For all assays, microsomes were rapidly thawed and mixed gently with the substrate solution. All microsomes were stored at −80 °C until used and were not subjected to more than two freeze-thaw cycles. All samples were prepared in triplicate with the resultant percent inhibition calculations based on the mathematical combinations for the differences in fluorescence between the test/test-blank

wells and the mean difference between each control and blank well. Thus, nine experimental values were achieved for each sample. Controls were run with every assay. All assays were performed under gold fluorescent lighting (Industrial Lighting, Ottawa, ON).

Testosterone assay

Extracts were analyzed on a XDB-C8 (4.6 × 150 mm, 5 µm particle size) Agilent 1100 series HPLC with a diode array detector. The flow rate was 1 ml/min and oven temperature was set at 37 °C using a linear gradient starting with an elution system containing acetonitrile: water (20 : 80 v/v) for 2 min, to a final system containing acetonitrile: water (80 : 20 v/v) at 8 min and maintained until 60 min. The peaks were detected using wavelengths of 238, 254 and 300 nm.

Enzyme hydrolysis

Selected capsule aqueous extractions were treated with 4.2 units of β-glucosidase (Sigma G-0395 from almonds and/or G-4634 from Brewers Yeast) in sodium acetate buffer (pH 5.0) overnight at 37 °C in a 1.5 ml microfuge tube with appropriate controls. After incubation, the samples were centrifuged for 18 min at 13,000 rpm and the supernatant was removed for testing with CYP 3A4.

P-Glycoprotein (P-gp) assay procedures

The P-gp ATPase analysis followed the method stated by GENTEST (1998) using verapamil (Sigma, lot # 56H0925) as a positive control measuring the orthovanadate-sensitive release of phosphate and ATPase activity using a THERMOmax Microplate Reader. All assays were performed under gold fluorescent lighting and samples were kept under reduced lighting conditions.

Results

A total of 18 products containing St. John's wort, including 7 caplets/tablets, 6 capsules, 4 teas (multiple lots) and 1 tincture, were studied (Table 1) for biomarker content and their ability to affect cytochrome P450-mediated metabolism of the marker substrates.

Since many of the reported pharmacologically active compounds are lipophilic, a series of solvents ranging in strength were used to extract the capsule material from NRP

Table 2. Quantity of active compounds found in the test products as determined by extractions in 70% acetone and 99% ethanol followed by HPLC analysis.

		Dry weight (mg)			
NRP	Form	% hyperforin	% hypericin	% pseudohypericin	% combined hypericins
8b	tea bags	0.67	0.018	0.02	0.038
8c	tea bags	0.144	0.022	0.038	0.06
93	tablets	0.502	0.063	0.12	0.183*
94	capsules	0.682	0.045	0.083	0.128
95	capsules	0.479	0.059	0.111	0.17*
96	tablets	2.699	0.051	0.187	0.238*
97	caplets	2.642**	0.065	0.097	0.162
98	capsules	0.039	0.046	0.13	0.176*
99	capsules	4.198	0.08	0.133	0.263*
100	tablets	0.039	0.008	0.017	0.025*
101	tincture[1]	2	3.2	1.4	4.6
102	capsules	0.068	0.052	0.083	0.135*
103	caplets	1.207	0.077	0.139	0.216*
104	tablets	0.589	0.039	0.069	0.108*
105a	tea bags	0.32	0.03	0.064	0.094
105b	tea bags	0.354	0.028	0.056	0.084
105c	tea bags	0.122	0.02	0.034	0.054
118a	tea bags	0.08	0.008	0.016	0.024*
118b	tea bags	0.068	0.01	0.016	0.026*
119b	tea bags	0.036	0.008	0.014	0.022
128	caplets	0.006	0.018	0.143	0.161*
129	capsules	2.013	0.057	0.132	0.189*

[1] Expressed in µg/ml.

* product reportedly standardized to 0.3% hypericin.

** product reportedly standardized to at least 3% hyperforin.

98 (Fig. 1). The first solvent extract in the series, hexane (yellow-green colour) followed by chloroform (brown-green), ethyl acetate (bright red), methanol extract (orange-red), 55% ethanol (light peach colour), and finally water (very faint peach colour). Extremely variable results were observed when testing the aliquots of ethyl acetate (169.9%) and hexane (157.0%) extracts against 3A4. The chloroform and methanol extracts also caused high inhibition with values of 97.6 and 87.5%, respectively, but the weaker solvents in this sequential extraction protocol, 55% ethanol and water, were less inhibitory (20.6 and 6.3%). A series of non-sequential extracts (data not reported) also found high activity in all extracts.

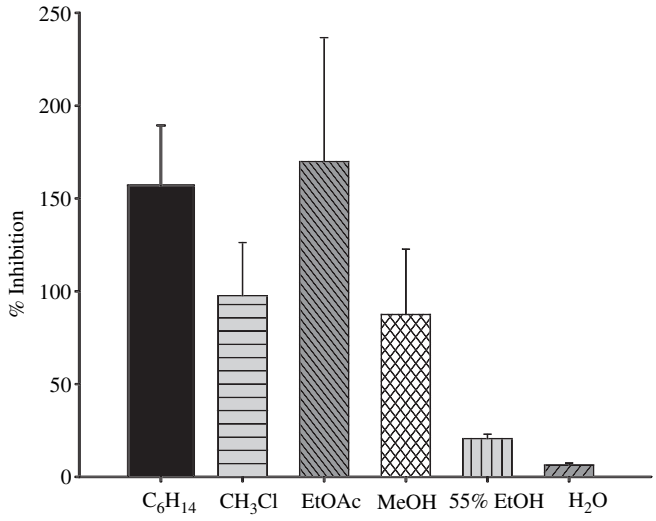

Figure 1. Inhibition of human cytochrome P450 3A4 marker substrate 7-benzyloxyresorufin metabolism by a series of solvent extracts of the St. John's wort NRP 98 (n = 3; 25 mg/ml stock solutions; % Inhibition ± SD).

Extracts from the 18 products were examined for hyperforin, hypericin, and pseudohypericin content (Table 2). Wide variation in content was found in all samples with the lowest levels being present in the teas. Although most products were reportedly standardized to either 0.3% hypericin or 3.0% hyperforin, many products did not meet the reported minimal content by our analysis. Three products (NRP 96, 97, 99) had a markedly higher hyperforin content than all other samples.

Aliquots of aqueous extracts of 11 capsule and tablet products were tested for their ability to inhibit the metabolism of cytochrome P450 2C9*1 and *2, 2C19, 2D6 and 3A4 isozyme marker substrates (Tables 3 and 4). All extracts markedly inhibited metabolism. The higher inhibitions were observed with the 2C isozymes; usually 2C19 being the highest followed by 2C9*2 and then 2C9*1. It was therefore necessary to reduce the sample concentration from 25 mg/ml to 5 mg/ml to prevent saturation. The inhibition results for the 2D6 and 3A4 (tested at 25 mg/ml) were very similar to each other and always greater than 50%. In a separate assay, aqueous extracts of the capsules were examined for their

Table 3. Inhibition of human cytochrome P450 isozymes by aliquots of aqueous extracts of St. John's wort capsules (n = 3; 5 mg/ml stock solutions except where noted; % Inhibition ± SD).

NRP	2C9*1	2C9*2	2C19	2D6[γ]	3A4[γ]
94	50.9 ± 14.61	64.4 ± 3.15	89.2 ± 6.31	67.8 ± 1.35	74.6 ± 2.13
95	57.4 ± 13.33	85.3 ± 2.03	100.9 ± 8.76	82.3 ± 0.66	88.5 ± 7.61
98	92.0 ± 4.17	90.2 ± 0.87	99.2 ± 7.49	85.7 ± 1.78	84.6 ± 5.36
99	71.9 ± 7.76	82.0 ± 1.82	79.9 ± 25.77	79.9 ± 6.64	71.4 ± 7.23
102	66.9 ± 13.18	85.2 ± 2.26	97.3 ± 5.71	75.8 ± 1.83	90.2 ± 3.96

[γ]25 mg/ml stock solution.

Table 4. Inhibition of human cytochrome P450 isozymes by aliquots of aqueous extracts of St. John's wort tablets (% Inhibition ± SD; 5 mg/ml stock solutions except where noted; n = 3 − 6).

NRP	2C9*1	2C9*2	2C19	2D6[γ]	3A4[γ]
93	90.0 ± 10.64	72.6 ± 3.09	78.5 ± 13.64	78.2 ± 1.29	51.1 ± 9.95
96	101.8 ± 2.98	84.0 ± 1.03	88.5 ± 13.00	84.1 ± 0.72	70.7 ± 7.50
97	61.0 ± 16.77	74.3 ± 1.45	90.8 ± 11.98	88.6 ± 2.17	80.1 ± 4.20
100	61.9 ± 4.08	40.3 ± 2.00	54.5 ± 12.59	54.2 ± 1.14	67.1 ± 5.58
103	77.3 ± 4.42	80.7 ± 2.14	87.0 ± 22.36	74.3 ± 1.83	64.4 ± 14.58
104	77.7 ± 11.72	70.7 ± 1.76	78.8 ± 22.24	76.5 ± 1.16	73.3 ± 18.46

[γ]25 mg/ml stock solution.

effect on 3A mediated metabolism with the 3A4, 3A5 and 3A7 isoforms (Table 5). All samples were inhibitory against the three isoforms with 3A5 having the lowest sensitivity to these extracts.

Aliquots of five aqueous extracts of the four teas were examined for their effect on 2C19 and two allelic variants of 2C9 and 2D6 (Table 6). All products were more inhibitory towards the 2C isoforms than against the 2D6 isoforms as previously seen with the capsules and tablets. Some inter-lot variability was noted with sample 8b against the 2C9 isoforms. These aqueous extracts were further examined for their effect against 3A4, 3A5 and 3A7 mediated-metabolism (Table 7). Inhibitory activity ranged from 31–94% against these isoforms. The 4 teas were also prepared as infusions to mimic the preparatory method in which the product would be ingested and then tested for their effect on 3A4 metabolism (Table 7). Similar inhibitory activity was found with the aliquots from the extracted samples of the four teas. In a final comparative study with these aqueous extracts, they were examined for their effect on 3A4 metabolism of testosterone to 6β-testosterone (Table 7). This testosterone assay is less sensitive than the fluorescent plate method but did show a similar inhibitory pattern.

Most plant constituents are conjugates which are generally unable to affect drug metabolism in this form. Aliquots of the extracts of the capsules were subjected to enzymatic hydrolysis and tested using the 3A4 isoform. The results obtained with the aqueous extracts incubated with β-glucosidase (Fig. 2) show little change in inhibitory effect except with NRP 102.

The tea infusions were also screened for their ability to affect the activity of the membrane protein P-glycoprotein relative to the positive control verapamil (Fig. 3). Compared to verapamil, a known inhibitor of P-gp ATPase, the inhibitory effects of the aqueous St. John's wort tea infusions were relatively low but detectable.

Table 5. Inhibition of human cytochrome P450 isozymes by aliquots of aqueous extracts of St. John's wort capsules (n = 3; 25 mg/ml stock solutions; % Inhibition ± SD).

NRP	3A4	3A5	3A7
94	65.0 ± 4.57	60.0 ± 3.23	94.8 ± 3.43
95	95.9 ± 6.42	26.9 ± 8.66	101.2 ± 3.99
98	69.7 ± 10.35	33.8 ± 28.49	101.0 ± 6.73
99	64.0 ± 9.31	45.7 ± 38.78	96.3 ± 5.14

Discussion

This study was initiated as less information was available on the effect of the highly inhibitory aqueous extracts on cytochrome P450-mediated metabolism and P-glycoprotein.

Table 6. Inhibition of human cytochrome P450 isozyme mediated metabolism by aqueous extractions of St. John's wort teas (n = 3; % Inhibition ± SD; 5 mg/ml stock solutions except where noted).

	2C9*1	2C9*2	2C19	2D6*1[γ]	2D6*10[γ]
NRP 8a	59.4 ± 8.59	61.4 ± 2.07	60.8 ± 20.73	33.9 ± 0.87	27.0 ± 1.59
NRP 8b	22.7 ± 7.86	22.9 ± 4.54	54.3 ± 35.99	25.0 ± 1.39	20.1 ± 2.48
NRP 105	85.3 ± 2.97	85.0 ± 2.50	92.6 ± 74.34	45.4 ± 4.34	40.3 ± 1.57
NRP 118	86.4 ± 4.91	90.3 ± 0.72	73.6 ± 27.12	59.3 ± 0.91	57.2 ± 0.29
NRP 119	80.8 ± 17.82	81.8 ± 1.64	57.8 ± 43.53	43.2 ± 0.78	39.0 ± 0.82

[γ] 25 mg/ml stock solution.

Table 7. Inhibition of human cytochrome P450 3A4, 3A5 and 3A7 marker substrate 7-benzyloxyresorufin metabolism by aqueous extractions of St. John's wort teas in the fluorescence plate assay and testosterone assay (in brackets) (n = 3; 25 mg/ml stock solutions; % Inhibition ± SD).

NRP	3A4[1]	3A4	3A5	3A7
8a	60.9 ± 0.60	62.6 ± 2.19	49.9 ± 23.71	84.0 ± 1.79
8b	58.6 ± 1.68	69.3 ± 4.76 (93.6)	51.2 ± 8.99	83.4 ± 2.26
105	70.9 ± 1.08	42.9 ± 2.41 (69.6 ± 0.06)	88.9 ± 14.31	75.8 ± 2.91
118	60.8 ± 1.35	93.0 ± 1.08 (65.2 ± 0.002)	67.6 ± 5.03	94.1 ± 2.42
119	60.1 ± 0.33	31.0 ± 1.11 (45.7 ± 0.24)	66.3 ± 6.91	91.7 ± 0.80

[1] Infusion 1 bag in 200 ml deionized water.

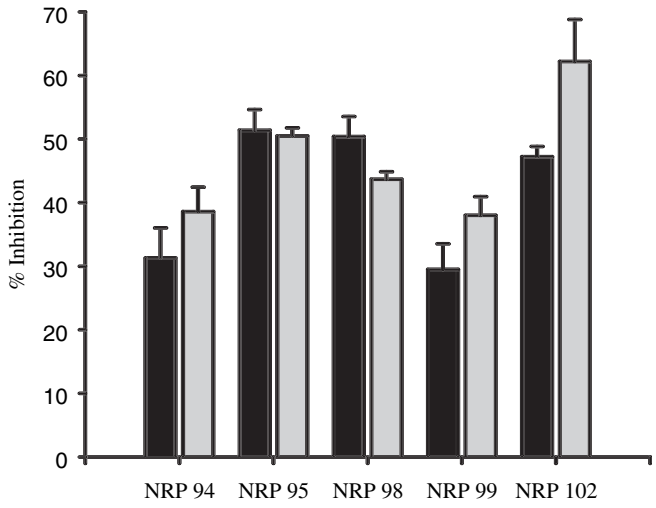

Figure 2. Inhibition of human cytochrome P450 3A4 marker substrate 7-benzyloxyresorufin metabolism by aliquots of aqueous extracts of St. John's wort capsules (25 mg/ml stock solutions) incubated at 37 °C overnight with and without β-glucosidase (n = 3; % Inhibition ± SD). With β-glucosidase (black), without β-glucosidase (grey).

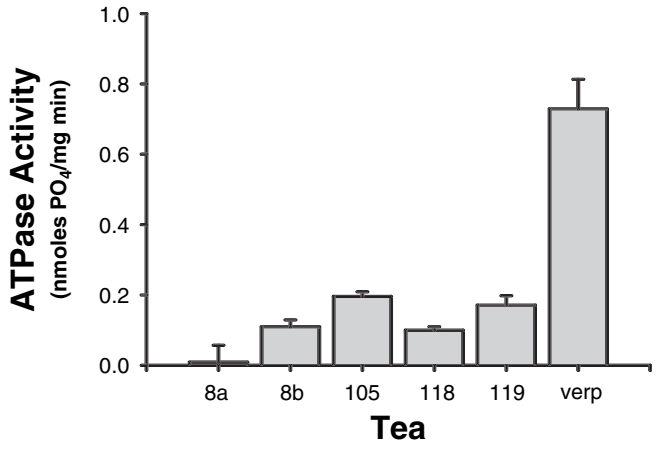

Figure 3. Inhibition of the ATPase activity of the P-glycoprotein membrane transport system by St. John's wort tea infusions (1 bag in 200 ml deionized water at 100 °C) as compared to the known inhibitor verapamil (20 µM, verp) (n = 3; nmoles PO_4/mg min ± SD).

A broad screen of 22 samples of 18 products was conducted to ascertain the constituent levels, and potential inhibitory effects of aqueous extracts of St. John's wort on the metabolism of marker substrates by cytochrome P450 isozymes and the ATPase activity affecting P-glycoprotein mediated transport. The results reported here are consistent with earlier our study with tinctures and the studies of Roby et al. (2000) and Obach (2000), that St. John's wort extracts could inhibit 3A4 mediated-metabolism. The findings from the sequential extractions revealed high inhibitory activity of several lipophilic extracts against the 3A4 mediated-metabolism. Interestingly, some inhibitory activity was recovered in the final aqueous extract. High activity was also found in non-sequential extracts. The findings with the ethanolic extracts in particular were consistent with published findings.

Aliquots of aqueous extracts of St. John's wort do not contain detectable amounts of the known biomarkers of *Hypericum*. It should also be noted that the inhibition results for the 3A4 with the aqueous capsule extracts were generally high, while the aqueous capsule extracts in the solvent series showed extremely low inhibition (6.3%). The reason for this difference is that the stronger solvents used earlier in the extraction series would have already removed the majority of the compounds present in the St. John's wort samples.

The aqueous capsule samples caused the most inhibition against 2C19, 2C9*1 and 2C9*2, and there was also marked inhibition (greater than 50%) of 3A4 and 2D6 metabolism. The tablets also exhibited marked inhibition of the 2C isozymes, and the majority of the tablet samples caused an inhibition of greater than 50% of the 2C19, 2C9*1, 2C9*2, 3A4 and 2D6 metabolism. Finally, the teas varied in the amount of inhibition of the 2C isozymes, but most of the inhibition values were lower than that of the capsules and tablets. In comparison, they also showed slightly lower inhibitions of the 2D6. In contrast to these findings, ethanolic extracts of St. John's wort were shown to be potent inhibitors of 2D6 metabolism (Obach, 2000).

Although the majority of the results for the aqueous capsule and tablet extractions showed high inhibitions, there were many results for the tea extractions which were quite different. The results for the 2C9*1 isozyme were the most dissimilar to all of the others. Many of these values suggested that the extracts were not inhibitory or slightly enhanced the metabolism of the 7BR. The 2C9*2 inhibition results were also quite different for the tea extractions when compared to the capsules and tablets. The tea samples tested with 2C9*2 showed low inhibition results, but no negative percentages. Since the two allelic variants of 2C9 showed different results for the aqueous tea samples, it is possible that they each reacted differently to some components within the tea. This difference between allelic variants however did not occur when testing the capsules and tablets. Considering this factor, and other variations between different samples, it is likely that there was a great deal of product variation. This emphasizes the importance of using a wide variety of samples to validate *in vitro* results.

The tests undertaken with the β-glucosidase were on the aliquots of aqueous extracts of several capsules. The majority of samples exhibited no significant change in inhibition after treatment with the enzyme. Some reasons for this is that there were simply no glycones present in the sample, or that no glycones were soluble in the aqueous extract. Another possibility is that the glycones were present but were not substrates of the β-glucosidase; or if they were substrates, their cleavage products may not have been inhibitory to 3A4.

P-Glycoprotein membrane proteins are transporters which can shuttle xenobiotics and or their metabolites in and out of the cells of the gut epithelium and the brain. P-Glycoprotein reacts differently depending on which particular xenobiotic is present. Like the cytochrome P450, this system can be induced or inhibited. In the case of the St. John's wort aqueous extractions, the P-gp was inhibited via the ATPase activity of each product by a low but measurable amount. These results suggest that it may be possible to use another stronger solvent to obtain higher inhibition results. Further studies on the subject should be done.

One of the major implications of the present studies involves the neonatal isozyme 3A7 which was found to be highly sensitive to inhibition by St. John's wort. Many label warnings along with the Natural Medicines database suggest that St. John's wort could be unsafe to take during pregnancy or lactation. These results substantiate warnings and suggest a strong possibility of St. John's wort being hazardous to neonates.

These inhibitory *in vitro* results with St. John's wort complements the inductive findings reported in the pharmacokinetic study with indinavir (Piscitelli et al., 2000) and heart transplant patients (Ruschitzka et al., 2000) in suggesting that caution should be exercised when combining St. John's wort and other therapeutic products or other natural products which are metabolized and transported by the same isozymes and transport system. Some examples of drugs which may interact in this biphasic fashion with St. John's wort to cause adverse side effects include pamitriptyline, antidepressants, barbiturates, cyclosporine, digoxin, narcotics, nonnucleoside reverse transcriptase inhibitors, nortriptyline, oral contraceptives, reserpine, photosensitizing drugs, protease inhibitors, and theophylline (Jellin, 2000; Nebel et al., 1999). If they share the same drug metabolism and transport proteins as St. John's wort, it is also possible that interactions with other herbals may cause side effects. As with all *in vitro* studies, the application of results are limited because of an inability to extrapolate the complexity of the human body without a follow-up of *in vivo* study. There are many possible factors which make each individual unique, including genetics, environment, health or disease, age, stress, diet and lifestyle products such as illicit drug, tobacco and alcohol.

The main focus of the current literature in this area is on the possible drug interactions involved with the 3A4 isozyme alone. The findings of this study suggest that drugs metabolized by many of the cytochrome P450 isozymes should be considered when studying interactions with St. John's wort. It is possible that a wide range of therapeutic products used in conjunction with St. John's wort could lead to adverse side effects.

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