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Chemical and pharmacological evaluation of *Hypericum perforatum* extracts[1]

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KEY WORDS *Hypericum perforatum*; High pressure liquid chromatography; serotonin uptake inhibitors; free radicals; antioxidants; hyperforin; hypericin; quercetin; rutin

ABSTRACT

AIM: To determine the concentrations of chemical characteristic to extracts of leaves and flowers of *Hypericum perforatum* (St John's wort) in a number of selected samples and, following chemical characterization, to investigate the effects of these extracts on several pharmacological properties including effects of the extracts on inhibition of 5-hydroxytryptamine (5-HT) uptake and on antioxidant properties. **METHODS**: The samples were analyzed for the presence of characteristic chemicals by high performance liquid chromatography (HPLC) directly coupled to ultraviolet wavelength absorbance and positive or negative mode electrospray mass spectrometric detection. The effects of extracts on 5-HT uptake were determined by quantifying ^{3}H-5-HT incorporation into rat hippocampal prisms. Estimates of effects of extracts on free radical scavenging capacity were made using a dynamic assay based on the ability of compounds to prevent the initiation of a colored reaction produced by the horseradish peroxidase catalyzed formation of hydroxyl free radicals from hydrogen peroxide using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) as the color indicator. **RESULTS**: The chemical profile of a number of extracts were determined and found to differ substantially from each other. Inhibition of 5-HT uptake was found to correlate with hyperforin content and free radical scavenging capacity was found to correlate with the content of several flavonoids including quercetin and hyperoside. **CONCLUSION**: Standardized extracts of *H perforatum* varied substantially in the concentration of several characteristic chemicals. The correlation between pharmacological activity and certain characteristic chemicals found in these extracts indicates that the medicinal benefit derived from selected extracts will vary considerably depending on their chemical composition.

INTRODUCTION

Hypericum perforatum L. (St John's wort) is a herbaceous perennial plant distributed in Europe, Northern Africa, and Asia and is now cultivated in North America. Extracts of the leaves and flowers of *H perforatum* have traditionally been used as sedative, diuretic, anti-inflammatory and wound healing agents and to relieve sciatica, hip pain, neuralgic pain, and melancholy[1]. Presently, *H perforatum* extracts are a popular treatment option for mild to moderate states of depression[2].

A number of chemical constituents of *H perforatum* including cinnamic acid derivatives (eg, chlorogenic acid), flavonoids (eg, rutin, hyperoside, quercetin, I3, II8-biapigenin), phloroglucinols (eg, hyperforin, adhyperforin, hydroperoxycadiforin) and naphthodianthrones (eg, hypericin, pseudohypericin), have known biological effects. For example: I3, II8-biapigenin is a known anti-inflammatory[3]; hyperforin has antibiotic effects[4] and inhibits the reuptake of several neurotransmitters[5]; and many flavonoids are effective antioxidants[6-8] and inhibitors of monoamine oxidase enzymes[9].

The naphthodianthrone, hypericin, was initially believed to be the chemical constituent of *H perforatum* that was responsible for antidepressant activity because it was shown to inhibit monoamine oxidase[10]. As a result, it

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became common practice to standardize *H perforatum* extracts to 0.3 % hypericin content. Further research, however, has indicated that constituents other than hypericin are likely to be responsible for the antidepressant effects of *H perforatum*[9,11-14]. Although the antidepressant efficacy of *H perforatum* has been demonstrated in several clinical studies[15-20], the mode of action of this herb has not been confirmed. A significant increase in 5-hydroxytryptamine concentrations in specific regions of rat brain has been shown following treatment with *H perforatum* extracts[21]. In addition, several flavonoids found in *H perforatum* extracts have been shown to inhibit monoamine oxidase *in vitro*[22,23], but the effect of these flavonoids on monoamine oxidase activity *in vivo* remains elusive. Studies also indicate that hyperforin is a relatively potent inhibitor of 5-hydroxytryptamine (5-HT), noradrenaline and dopamine (DA) uptake[5,14]. These findings suggest that a number of chemicals found in *H perforatum* extracts may contribute to antidepressant effects and that they may act via different mechanisms. Thus, precise chemical characterizations of extracts from *H perforatum* are necessary before forming conclusions based on pharmacological evaluations.

In general, analysis of most compounds found in *H perforatum* can be accomplished using gradient elution reverse phase high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) absorbance detection between 240 and 280 nm. Separations designed to resolve all of the cinnamic acid derivatives, flavonoids, naphthodianthrones and phloroglucinols require extended run times, often up to 60 min, making analysis unwieldy[24]. Alternatively, rapid separations designed to accurately quantitate naphthodianthrones and phloroglucinols often preclude quantitation of the relatively more polar flavonoids and cinnamic acid derivatives since these chemicals elute in the solvent front. Furthermore, the napthodianthrones produce poor absorbance signals in this wavelength range (ie, 240 to 280 nm) and absorbance measurements in the visible range (eg, 590 nm) are more sensitive. Thus, use of a scanning diode array detector with a range from about 190 to 950 nm is preferable.

Most of the chemicals, with the exception of the naphthodianthrones, found in *H perforatum* can be analyzed by means of conventional positive mode electrospray mass spectrometry (MS), but they produce relatively weak signals. Although negative mode electrospray MS for analysis of cinnamic acid derivatives, flavonoids, naphthodianthrones and phloroglucinols in *H perforatum* extracts has rarely been used, preliminary work suggests that negative mode electrospray analysis of *H perforatum* extracts may be superior to positive mode procedures[24].

The chemical characterization of *H perforatum* extracts permits the evaluation of relationships between pharmacological effects and specific chemicals or groups of chemicals identified in these extracts. For example, correlations between uptake of neurotransmitters or free radical scavenging activity and specific chemicals or groups of chemicals (eg, hyperforin, hypericin and flavonoids) found in *H perforatum* extracts can be established. This paper will summarize the correlations found in some initial studies.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (200 – 250 g; BioScience Animal Services, Ellerslie, Alberta, Canada) were housed 2 per cage and maintained on a 12-h light/dark cycle (lights on at 6:00 am; 22 ℃) with *ad libitum* access to food and water. All animal procedures were in accordance with the regulation of the Canadian Council on Animal Care.

Herb and chemical sources *Hypericum perforatum* extracts reportedly standardized to 0.3 % hypericin content were obtained from several commercial sources. To provide a wide range of sample quality, several other extracts containing substantially different quantities of hyperforin and hypericin were provided by our research facilities. The identity of each *H perforatum* extract was confirmed using HPLC coupled to UV absorbance and electrospray mass spectrometric detection to determine the presence of rutin, hyperoside, I3, II8-biapigenin, hypericin, hyperforin, and other characteristic chemical components.

Most chemical reagents were purchased from either Sigma-Aldrich Chemical Co, Oakville, Ontario, or Fisher Chemical Co, Edmonton, Alberta. Hyperoside was purchased from Indofine Chemical Co, Belle Mead, New Jersey. Hypericin and hyperforin were purchased from ChromaDex Inc, Irvine, California. Radiolabelled [1, 2-^{3}H[N]] 5-hydroxytryptamine creatinine sulfate (1110 TBq/mol) was purchased from New England Nuclear, Boston, Massachusetts. Solvents were HPLC grade.

HPLC and mass spectrometry Chromatographic separation of constituents of *H perforatum* extracts was accomplished on a Hewlett Packard 1090 HPLC system equipped with a G1315A diode array detector using two methods. The first method, used to quantitate

naphthodianthrones and phloroglucinols, used an isocratic separation on a reverse-phase column. The mobile phase consisted of 50 % acetonitrile, 30 % methanol, and 20 % 0.1 mol/L ammonium formate and the flow rate was 1.5 mL/min. The column was a Phenomenex Luna C_{18} (25 cm × 4.1 mm ID, 5 μm particle size) and eluting peaks were monitored by following UV/vis absorbance between 190 nm and 950 nm. The second method, primarily for quantitation of cinnamic acid derivatives and flavonoids, used a binary gradient elution on a reverse-phase column. Mobile phase A was 5 % acetonitrile and 0.1 % trifluoroacetic acid and mobile phase B was 95 % acetonitrile and 0.1 % trifluoroacetic acid. The gradient conditions were as follows: 0 % B to 100 % B over 40 min, 100 % B for 5 min, 100 % B to 0 % B over 5 min, 0 % B for 5 min, at a flow rate of 1 mL/min. The column was a Phenomenex C_{18} (25 cm × 4.1 mm ID, 5 μm particle size) and eluting peaks were monitored by following UV absorbance at 254 nm.

A Hewlett Packard 1050 HPLC system equipped with a UV absorbance detector coupled directly to a Fissons VG Quattro electrospray mass spectrometer was used for electrospray MS. After UV absorbance detection at 254 nm, material eluting from the column was split so that 50 μL/min was fed directly into the mass spectrometer. Mass signals between mass 200 and 1000 were monitored in positive or negative ion mode using a cone voltage of 30 V. Eluting peaks that did not provide strong electrospray signals (eg, hypericin and hydroperoxycadiforin) were often individually collected from the outlet of the flow splitter, freeze dried and re-evaluated by direct injection into the electrospray apparatus in both positive and negative mode. For positive mode analysis, material used for direct injection was dissolved in acetonitrile: 0.1 % aqueous solution of trifluoroacetic acid (1:1). For negative mode analysis, the material was dissolved in acetonitrile: 10 mmol/L aqueous ammonium hydroxide solution (1:1).

Tandem electrospray MS was used to evaluate several compounds collected from eluting samples. Eluting peaks from samples were collected, freeze dried, and reconstituted in 100 μL of a mixture of acetonitrile and 0.1 % aqueous solution of trifluoroacetic acid (1:1) prior to direct injection onto the mass spectrometer. Daughter ions of selected protonated signals (eg, m/z 537, hyperforin) were recorded in both the absence and presence of argon collision gas (cone voltages ranged from 5 to 40 V).

Free radical scavenging capacity Hydroxyl free radical scavenging ability of *H perforatum* extracts and pure chemicals was assessed by a modification of the dynamic method developed by Arnao, *et al*[25], with 50 mM tris-HCl buffer (pH 7.2) and 2 nmol/L horseradish peroxidase. The dynamic method was chosen over that of the single point methods described by Miller *et al*[26-28] and Salah *et al*[29] after it was determined that a number of plant extracts and flavonoids inhibited the peroxidase reaction. This inhibition can lead to an erroneous estimation of the hydroxyl free radical scavenging capacity of chemicals that are peroxidase inhibitors and/or free radical scavengers if a single point assay is used.

5-Hydroxytryptamine (5-HT) reuptake Effects of *H perforatum* extracts on 5-HT reuptake were determined *in vitro* in rat hippocampal prisms with [^{3}H]5-HT as the substrate and according to the procedure[30].

RESULTS

Extracts of *H perforatum* provided chromatographic profile characteristic of each preparation. Fig 1 illustrates a representative UV absorbance HPLC trace obtained from an extract of *H perforatum* using the isocratic method designed to quantify hypericin and hyperforin. This method provides separation of all major naphthodianthrones and phloroglucinols within 20 min and is compatible with negative mode electrospray MS analysis. Fig 2 illustrates a representative UV absorbance HPLC trace obtained from an extract of *H perforatum* using the method designed to measure cinnamic acid derivatives and flavonoids as well as naphthodianthrones and phlorogluci-

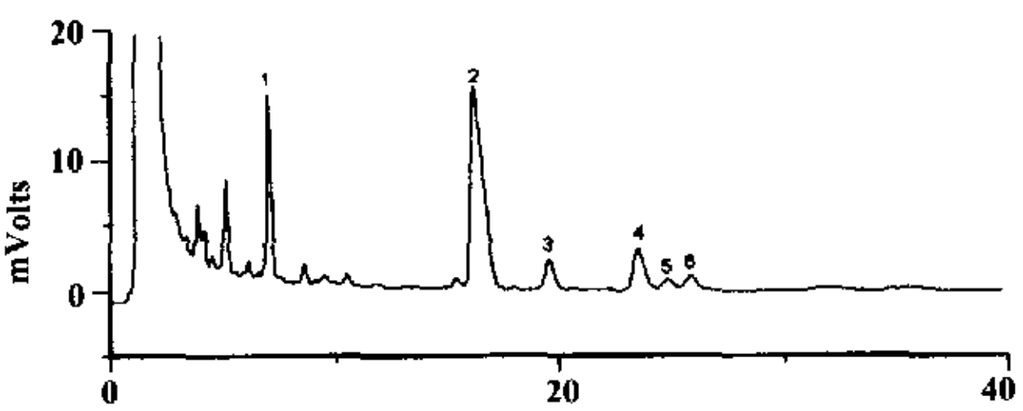

Fig 1. Representative chromatogram of an extract of *H perforatum* separated by an HPLC method designed to resolve naphthdianthrones and phloroglucinols. Ultraviolet absorbance is at 254 nm. Peaks are pseudohypericin (1), hyperforin (2), adhyperforin (3), hypericin (4), isomer of hyperforin (5), and isomer of adhyperforin (6). Mass spectral analysis indicates traces of additional isomers of hyperforin and adhyperforin eluting at about 32 and 36 min, respectively.

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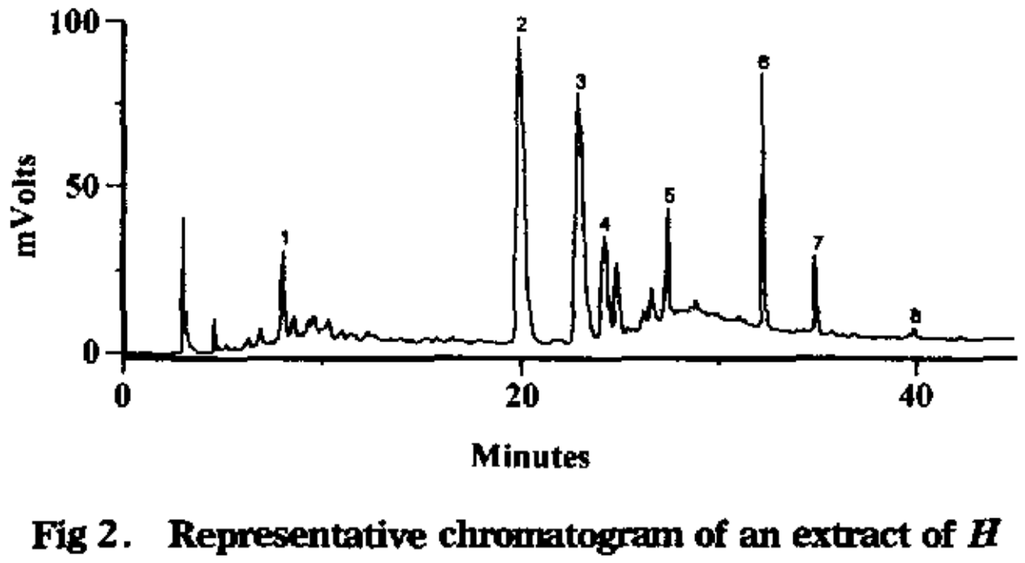

Fig 2. Representative chromatogram of an extract of *H perforatum* separated by an HPLC method designed to resolve cinnamic acid derivatives, flavonoids, naphthodianthrones, and phloroglucinols. Ultraviolet absorbance is at 254 nm. Peaks are chlorogenic acid (1), rutin (2), hyperoside (3), isoquercitrin (4), quericitrin (5), quercetin (6), I3, II8-biapigenin (7), and pseudohypericin (8).

nols. This separation takes about 60 min, but is advantageous because the mobile phase is acidic and volatile, thus, ideal for positive mode electrospray MS analysis.

The positive mode electrospray total ion trace (not shown) provides strong signals for the phloroglucinols in addition to relatively weak, but characteristic signals for the cinnamic acid derivatives and flavonol glycosides. No electrospray signals were obtained for the naphthodianthrones in the positive mode. Representative positive mode mass spectra of 4 flavonoids (rutin, hyperoside, quericitrin, and I3, II8-biapigenin) obtained from electrospray traces are illustrated in Fig 3. The quercetin glycoside, rutin, was identified by both its retention time and characteristic mass spectrum, which provided signals at MNa^+ (m/z 633), MH^+ (m/z 611) and fragments at

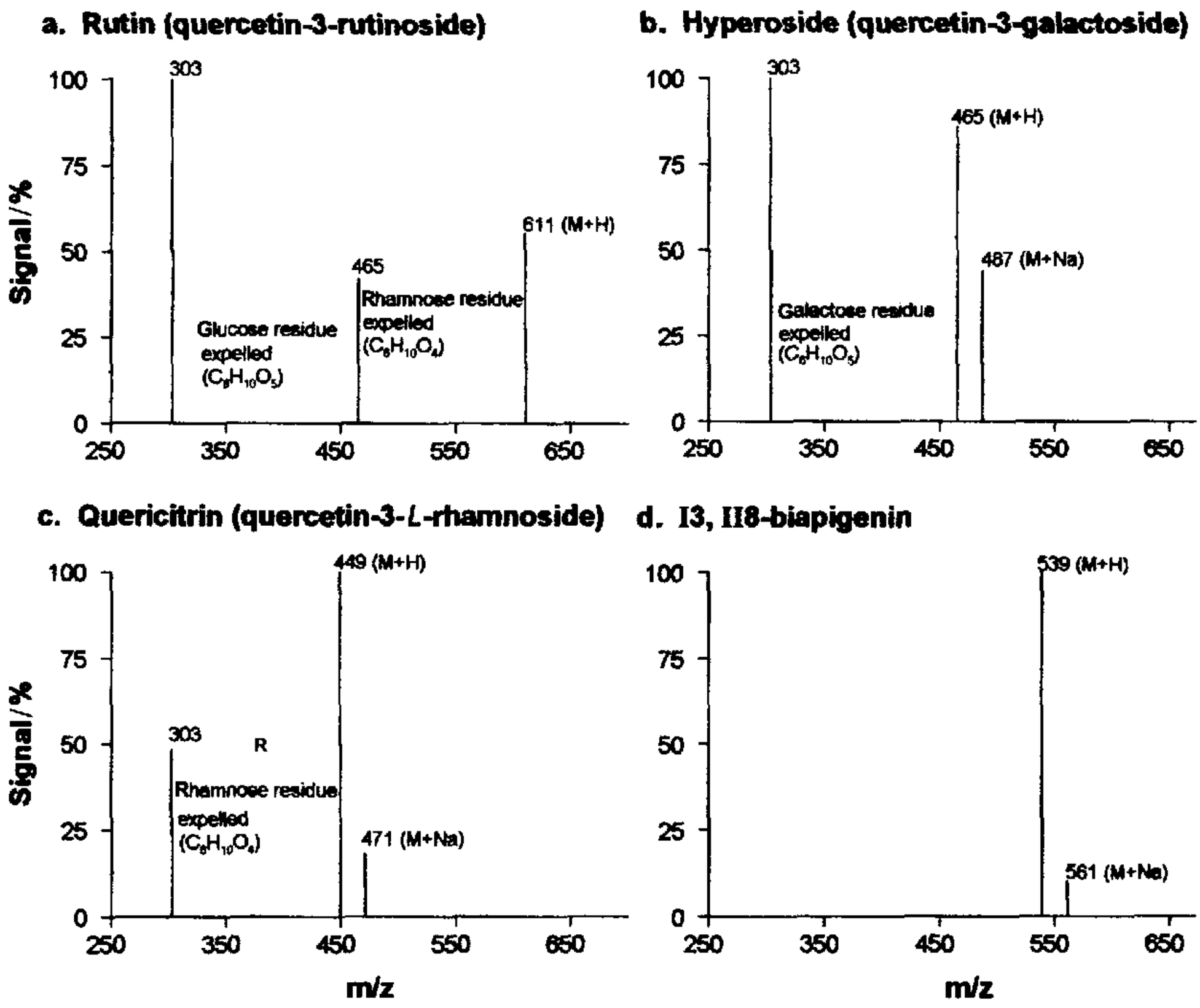

Fig 3. Representative positive mode electrospray mass spectra of rutin (a), hyperoside (b), quericitrin (c), and I3, II8-biapigenin (d) from the *H perforatum* extract illustrated in Fig 1. The HPLC eluant from this extract was fed directly into the MS. Some fragmentation due to expulsion of sugar residues is observed with the flavone glycosides.

m/z 465, m/z 303 (quercetin) and m/z 265. Hyperoside (quercetin-3-*O*-glucoside) was identified by its retention time and by mass spectrum signals at MNa^+ (m/z 487), MH^+ (m/z 465) and a fragmentat m/z 303. Quericitrin was identified by its retention time and by mass spectrum signals at MNa^+ (m/z 471), MH^+ (m/z 449) and a fragment at m/z 303. I3,II8-biapigenin was identified by mass spectrum signals at MNa^+ (m/z 561) and MH^+ (m/z 539).

Positive mode MS/MS provided informative characteristic fragmentation patterns for several phloroglucinols. Fig 4 illustrates a positive mode tandem electrospray mass

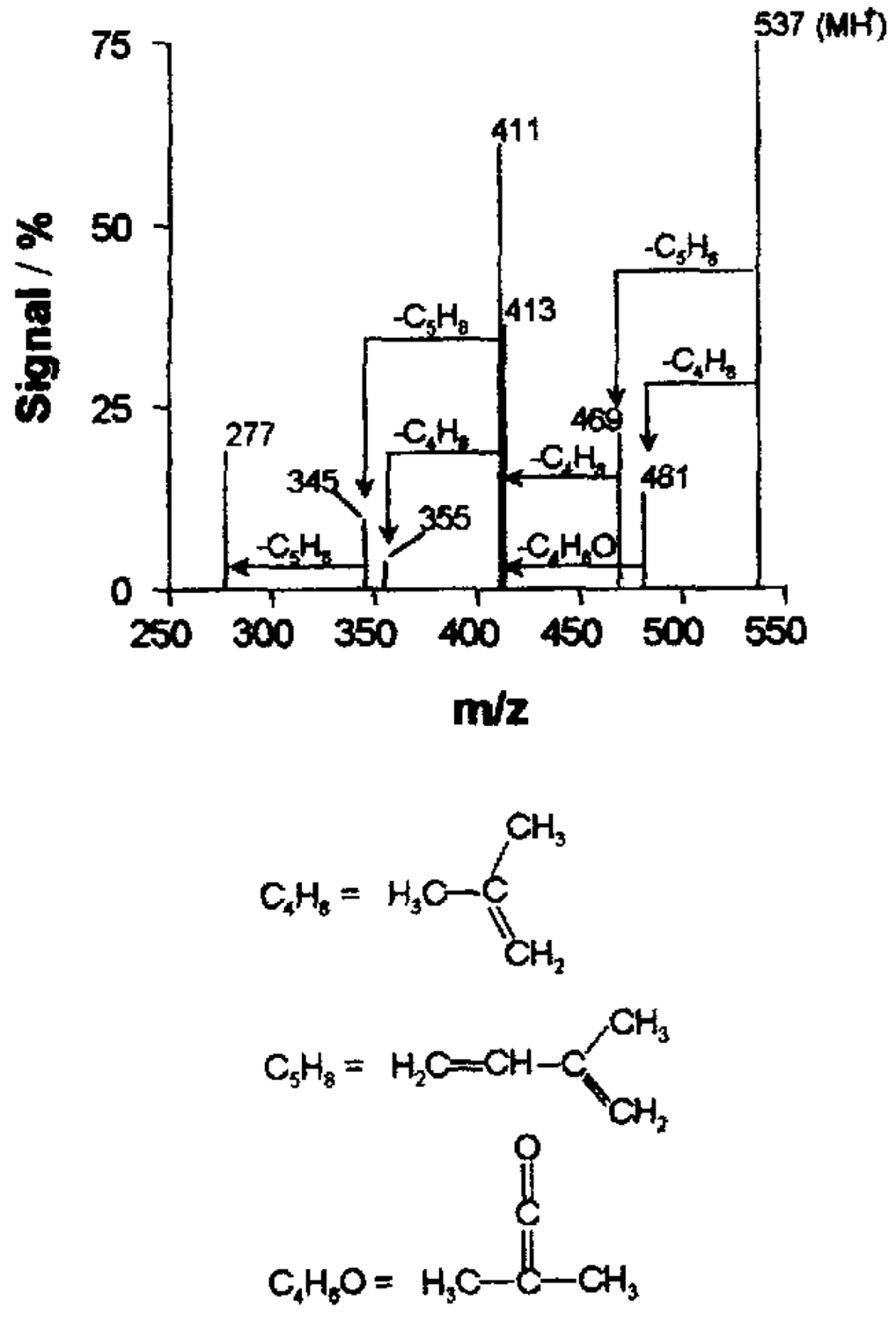

Fig 4. Representative positive mode tandem electrospray mass spectrum obtained from hyperforin isolated and concentrated from an eluting peak derived from the separation illustrated in Fig 1. The intensity of the MH^+ signal has been arbitrarily set to 75 % to better illustrate the fragments. Fragmentation caused by sequential loss of the side chains is demonstrated.

spectra analysis of hyperforin obtained by collecting the eluting peak of interest from the outlet of the flow splitter, concentrating by freeze-drying and injecting it directly into the electrospray unit. Little or no fragmentation was observed in the absence of argon. In the presence of argon certain compounds (eg, hyperforin) provided strong fragmentation spectra. Fragmentation patterns obtained from hyperforin (daughters of m/z 537) in the presence of argon with a collision energy of 30 V include m/z 481, m/z 469, m/z 413, m/z 411, m/z 355, m/z 345, and m/z 277. It should be noted that hyperforin is unstable in acidic environments including those used for positive mode electrospray MS which contain 0.1 % trifluoroacetic acid, and analysis should be performed immediately after reconstitution in such solutions. Negative mode MS/MS provided rather weak fragmentation patterns when compared to positive mode MS/MS.

The flavonoids (ie, quercitin, hyperoside and rutin), hypericin, and hyperforin contents of 16 *H perforatum* extracts are shown in Tab 1 (extracts 1 – 10 are

Tab 1. Content of hyperforin, hypericin, and individual flavonoids in *Hypericum perforatum* extracts.

Extract number	Hyperforin (mg/g)	Hypericin (mg/g)	Rutin (mg/g)	Hyperoside (mg/g)	Quercetin (mg/g)
1	24.10	0.49	19.32	37.91	7.86
2	19.11	0.48	18.63	37.16	7.93
3	13.31	0.32	21.54	35.87	5.23
4	2.25	0.27	27.01	14.47	2.76
5	12.96	0.34	27.14	28.53	3.16
6	7.51	0.38	39.34	31.90	6.69
7	4.59	0.45	27.04	25.35	3.45
8	0.84	0.00	1.79	3.34	1.49
9	0.67	0.30	12.01	10.93	1.00
10	0.72	0.29	10.96	10.03	0.91
11	0.00	0	61.84	1.51	2.25
12	6.50	1.72	33.12	4.07	1.11
13	303.20	0.00	0	0	0
14	4.30	1.43	17.94	3.42	0.30
15	43.79	3.12	1.60	2.52	0.46
16	10.30	2.06	4.47	1.96	5.67

*Extracts 1 – 10 were commercially obtained and each claimed to contain 0.3 % (ie, 3 mg/g) hypericin.

claimed to be standardized to 0.3 % hypericin). The free radical scavenging capacity correlated positively to quercetin content (Fig 5) and hyperoside content (data not shown). Despite the positive correlations, it is obvious that other components also contribute to free radical scavenging capacity because the regression line did not pass through the origin in either of these analyses. There was no correlation between free radical scavenging capacity and hyperforin or hypericin content, but an interesting observation was that one sample with a high hyperforin

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content and no flavonoids had no free radical scavenging capacity.

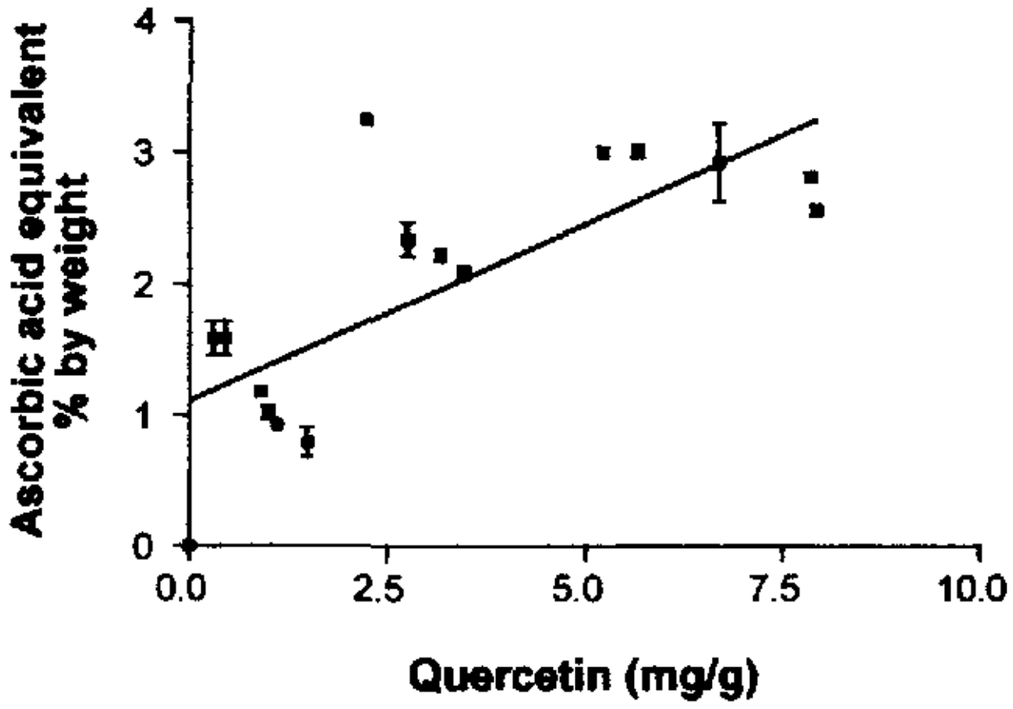

Fig 5. Relationship between quercetin content and free radical scavenging capacity of 16 *H perforatum* extracts. Free radical scavenging correlates positively with quercetin content, but because other materials (possibly flavonol glycosides, tannins and ascorbate) contribute, the X-intercept of the regression line is at approximately 1 % ascorbic acid equivalent.

The *in vitro* IC_{50} values of 6 *H perforatum* extracts as inhibitors of 5-HT reuptake are shown in Tab 2. The content of hyperforin, hypericin, and some individual flavonoids in these extracts is shown in Tab 1. A positive correlation between inhibition of 5-HT reuptake and hyperforin content was determined by plotting single data points (ie, % inhibition of reuptake at one concentration) for each extract versus the actual hyperforin content at that concentration (Fig 6). These data indicate that the ability of the extracts to inhibit 5-HT reuptake is not completely dependent on hyperforin content and that other constituents in the extracts are also active in this regard. There was no correlation between 5-HT reuptake and hypericin content or flavonoid content.

Tab 2. Effects of six *H perforatum* extracts on the reuptake of 5-HT into rat hippocampal prisms.

Extract number	IC_{50} (mg/L)
11	20.80
12	60.10
13	0.77
14	452.00
15	7.40
16	24.00

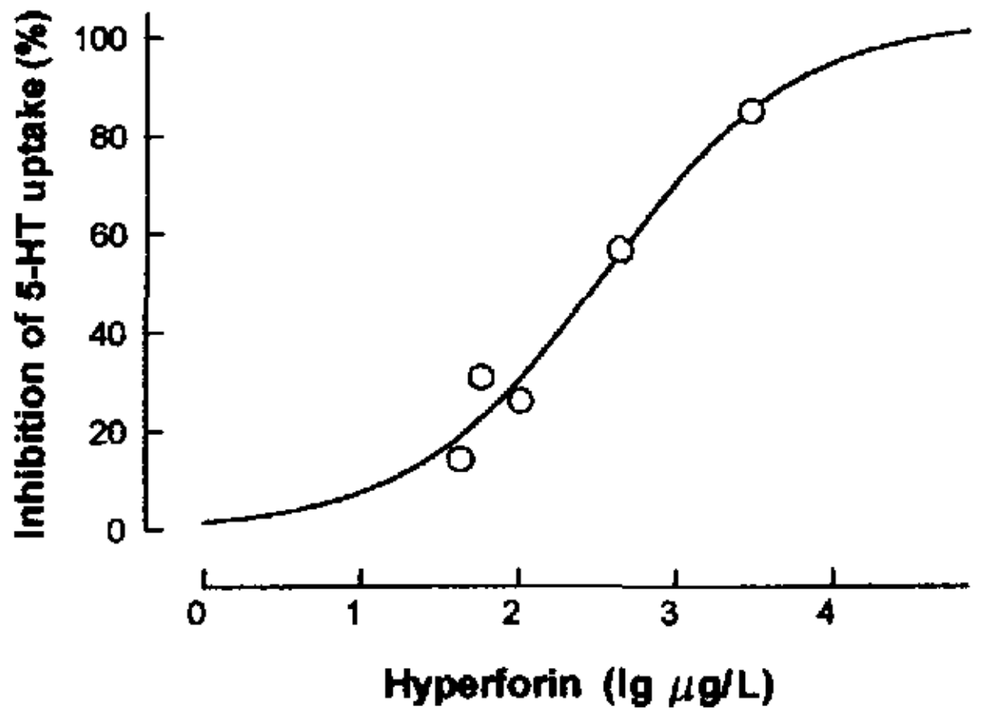

Fig 6. Relationship between hyperforin content and inhibition of 5-HT reuptake by five *H perforatum* extracts. Data are from 5 different extracts with calculated actual concentrations of hyperforin at the same data point (ie, 10 mg/L). The IC_{50} value for hyperforin at this concentration was 349 μg/L (95 % CI; Hill slope = 0.7 ± 0.3).

DISCUSSION

The continuing and expanding use of *H perforatum* extracts necessitates the need for specific methods to chemically and pharmacologically characterize the individual components. *H perforatum* extracts contain at least three groups of chemicals that may have beneficial antidepressant effects. Evidence suggests that hyperforin and related phloroglucinols inhibit neurotransmitter uptake[5,14,31]; flavonoids act as benzodiazepine receptor ligands[32], antioxidants[6], and monoamine oxidase inhibitors[22]; and hypericin and related naphthodianthrones inhibit monoamine oxidase[10], although this latter point has been disputed[9,11,12]. The diversity of chemical constituents and pharmacological activity of *H perforatum* extracts indicates that there is a large potential for inconsistent efficacy in products. For example, *H perforatum* extracts containing high amounts of hyperforin would be expected to act through different mechanisms and with different efficacy than extracts containing high amounts of flavonoids. It is obvious from our studies that, although extracts may claim to be standardized to a marker chemical (eg, 0.3 % hypericin), there are large chemical and pharmacological variations between extracts.

The two HPLC methods presented in this paper permit a sensitive and accurate evaluation of the major cinnamic acid derivatives, flavonoids, naphthodianthrones and phloroglucinols found in *H perforatum* extracts. The identity of these compounds is supported by electro-

spray MS in both positive and negative ion mode. The quantities of individual compounds (especially hypericin and hyperforin) found in *H perforatum* extracts varied widely, with some extracts being completely devoid of some characteristic constituents. It was also shown that the pharmacological activity of *H perforatum* extracts may correlate with the content of selected characteristic chemicals. These findings support the investigations of others[33,34,14]. Inhibition of 5-HT uptake was shown to have a positive correlation with the hyperforin content of the extract. In agreement with Chatterjee, *et al*[14], however, the data indicate that other constituents present in the extracts are either active or may act to modulate the activity of hyperforin. As hyperforin content can range from about 30 % to nil depending on the method of extraction, see[31,33] for examples of extraction procedures that result in different concentrations of hyperforin and, consequently, different pharmacological profiles), the expected benefit derived from inhibition of monoamine uptake may vary accordingly.

In conclusion, *H perforatum* extracts contain a number of potentially effective antidepressant chemicals. Our investigations demonstrate that different *H perforatum* extracts (even those standardized to 0.3 % hypericin) can vary substantially in the quantity of these chemical components and can have different pharmacological profiles. These studies demonstrate the necessity for knowledge of the chemical profile of particular *H perforatum* extracts when performing pharmacological investigations. Such studies would generate a greater understanding of the medicinal benefit of herbal extracts and establish guidelines to ensure consistent and effective products for the consumer.

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