

Full Length Research Paper

Composition and antioxidant activity *in vitro* of different St. John's Wort (*Hypericum perforatum* L.) extracts

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Current literature reports a wide variability in active compounds content in marketed *Hypericum* (*Hypericum perforatum* L.) preparations. In order to avoid influences by soil, climate, collection time and storage, several extracts were prepared, with different solvents, from a single stock of plant drug, collected from the wild. They were high performance liquid chromatography (HPLC) analyzed, and results were coupled with an evaluation of the antioxidant activity *in vitro* of each extract. Also water extracts maintained a good activity, although they were obviously lacking hypericin and hyperforin. In this sense, the home made St. John's wort teas should be re-valued as possible beverages with interesting antioxidant properties.

Key words: Antioxidant activity, flavonoids, hyperforin, hypericin, *Hypericum perforatum* L., St. John's wort tea.

INTRODUCTION

St. John's wort (*Hypericum perforatum* L. – Guttiferae) is a perennial herb spontaneous in Europe, Asia and North Africa, and introduced, naturalized, in North America and temperate areas of the southern hemisphere (Huxley et al., 1992; WHO, 2002), which has been used since ancient times by many populations for medicinal purposes. The most common use regards the flowering tops, an oily extract of which is a common popular remedy against wounds and burns. This use was recently re-examined by Süntar et al. (2010): they conclude that "*H. perforatum* possesses a remarkable wound healing activity supporting the folkloric assertion of the plant in folk medicine". A new interest arose on this species in the eighties of last century, when a true antidepressive activity was discovered for this plant drug, still the flowering tops (Bombardelli and Morazzoni, 1995; WHO, 2002; Ph.Eur., 2010; Süntar et al., 2010). Although there is not a general consensus of opinion on the chemical constituents (hypericin, hyperforin, flavonoids) mainly responsible for this activity (Bombardelli and Morazzoni, 1995; Chatterjee et al., 1998; Butterweck et al., 2000; Cervo et al., 2002; WHO, 2002; Butterweck et al., 2003; Paulke et al., 2008), the plant drug and many kinds of its

extracts are widely utilized, even in the clinical practice, and actively put on the market. Since the inclusion of this species in two lists of plants with antioxidant/radical scavenging properties published by Bol'shakova et al. (1997, 1998) and two contemporaneous papers by Tripathi and Pandey (1999) and Tripathi et al. (1999), also this activity has been usually claimed by medicinal plant sellers, although deeper studies were performed only in successive years. In fact, this activity has been more and more accurately investigated, for both *H. perforatum* (Sloley et al., 2000; Hunt et al., 2001; Benedí et al., 2004; Zou et al., 2004; Di Paola et al., 2007; Sánchez-Reus et al., 2007; Sagrantini et al., 2008; Filippini et al., 2010) and many allied species of the same genus, in that the number of published studies strongly increased in recent years. Furthermore, the antioxidant properties are generally considered colligated to a possible anticancer activity, and this gave rise to new research strategies (Couladis et al., 2002; Conforti et al., 2007; Dell'Aica et al., 2007; Drongre et al., 2008). In particular, Conforti et al. (2007) underline the strong cytotoxic activity exhibited by I3,II8-biapigenin.

There are no doubts that the chemical structures (mainly phloroglucinols and polyphenols) of many constituents of St. John's wort possess and easily explain antioxidant properties; however these structures are very different from one another, and confer to single molecules different features, for example, extractability.

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This means that the various extracts got from the market or extemporaneously prepared before consumption do not contain the whole group of phytoconstituents of *H. perforatum*, but only some of them, and this obviously reflects on the pharmacological profile of single extracts. Differences due to extraction methods are at the base of many reported problems for the use of *Hypericum* preparations in the pharmaceutical field, as well as problems due to instability in time or to light exposure of some of the active constituents (Wagner and Bladt, 1994; Constantine and Karchesy, 1998; Wurglics et al., 2001; Bergonzi et al., 2001; Draves and Walker, 2003; Ramanauskienė et al., 2004; Shah et al., 2005). For instance, many of the products put on the market are standardized, but not always in the same component. Also some of the papers published on *Hypericum* antioxidant activity only refer to a single group of constituents (Valentão et al., 2002, 2004; Zou et al., 2004; Sánchez-Reus et al., 2007).

The aim of the present study was therefore to verify and compare the effective antioxidant activity of several St. John's wort extracts, prepared with different methods and solvents. All the extracts were analyzed by HPLC to compare them in regard to their chemical compositions. Also water extracts, recalling the home-made *Hypericum* teas, were prepared, although it is commonly recommended not to use teas since the active principles hypericin and hyperforin do not pass in water extracts, not considering that other phytoconstituents, as flavonoids in the glycosidic form, are however water-soluble. For instance, out of the three studies on the antioxidant properties of the allied species *H. androsaemum* L., two were performed on extracts obtained with hot water, while the third on a hydroalcoholic extract (Valentão et al., 2002, 2004; Almeida et al., 2009). In these papers the activity of the extracts was attributed only to polyphenols.

MATERIALS AND METHODS

Plant material

Since environmental factors (soil, exposure, rain, temperature and so on) deeply influence the chemical composition of this plant drug (Bombardelli and Morazzoni, 1995; Pietta et al., 2001; Southwell and Bourke, 2001; Kazlauskas and Bagdonaitė, 2004; Smelcerovic and Spittler, 2006), as well as the collected plant parts and the developmental stage (Zobayed et al., 2006; Xenophontos et al., 2007; Filippini et al., 2010), all plant material for this study was contemporaneously collected from a single population growing on the Amiata Mountain (Grosseto province, Southern Tuscany, Italy), at about 1,000 m above sea level. Flowering tops were collected in the morning at the end of July, when flowers in anthesis and young green fruits were contemporaneously present, according to the recommendations by Pietta et al. (2001), because of the higher content of hypericin in the flowers and of hyperforin in fruits. For a detailed study of storage dynamics of different metabolites, which reach the peak at different biological/developmental stages (Filippini et al., 2010).

Plant drug was then dried in the dark at room temperature, to

avoid possible degradation of sensible constituents, preserved under the same conditions and powdered immediately before use.

Plant material extraction

Crude drug was powdered in a IKA A 10 (IKA® Werke GmbH, Staufen, Germany) knife-mill, sieved and subsequently extracted.

Five extracts were prepared, each with 5 g of powdered and sieved crude drug and 50 ml of solvent. Solvents had different polarities, and various extraction conditions (time of contact and temperature) were used, to obtain the following products:

(I) Methanolic extract: with methanol in ultrasonic stirring bath (Biosonik III – Bronwill Scientific Inc., Rochester, NY, USA) for 1 h at room temperature.

(II) "Pre-treated" methanolic extract: after a pre-treatment with dichloromethane in ultrasonic stirring bath for 1 h at room temperature to remove waxes and chlorophyll components (with this treatment also some pharmacologically active components could be removed), the obtained extract was filtered and the residue dried under vacuum to constant weight, then extracted again with methanol in ultrasonic stirring bath for 1 h at room temperature (Franchi et al., 1999, 2001, 2005).

(III) Hydroalcoholic extract: with ethanol at 40° in ultrasonic stirring bath for 3 h at room temperature.

(IV) Aqueous extract at room temperature: with distillate water in ultrasonic stirring bath for 3 h.

(V) Decoction: with distillate water under reflux for 15 min. The use of a decoction, undoubtedly a stronger method than infusion, is recommended by WHO (2002), although in this way most sensible molecules could be degraded.

The solvents were successively removed under vacuum to obtain the dried extracts (I → V), utilized in the following tests.

Spectrophotometric evaluation of antioxidant activity

The antioxidant activity of the five extracts was evaluated spectrophotometrically, using a method based on the photo-oxidation of *o*-dianisidine sensitised by riboflavin (Chopra et al., 1989; Franchi et al., 1999, 2001, 2005). In this assay free radicals are generated through *o*-dianisidine photo-oxidation: a general free radical scavenging compound has an inhibitory effect on this reaction, leading to a decrease in the final product, the oxidised *o*-dianisidine, measurable spectrophotometrically at 460 nm. The solution (1.3×10^{-5} M) of riboflavin (Sigma-Aldrich, St. Louis, MO, USA) was prepared in phosphate buffer 0.01 M pH 7.5; the solution (10^{-2} M) of *o*-dianisidine dihydrochloride (Sigma-Aldrich) in Milli-Q (Milli-Q plus – Millipore, Billerica, MA, USA) distilled water.

The extracts were dissolved in suitable solvents up to a final tube concentration between 0.58 and 18.0 µg/ml. The test was performed according to Chopra et al. (1989), each sample was treated at least in triplicate. The final product of photo-oxidation was measured with a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) at 460 nm. The inhibition percentage (I%) was calculated for each concentration of each extract in respect to its proper control (which was ascribed I% = 0); data were expressed as IC₅₀ values (concentration inhibiting of 50% the oxidised *o*-dianisidine formation in comparison to control). Result evaluation was carried out with EasyFit 1.4 (Matteo Vaccari and Mario Negri Institute, Milan, Italy). In order to better understand scavenging ability of extracts, and to have references for comparison purposes, a group of five pure compounds (glutathione, captopril, rutin, quercetin, catechin, all purchased from Sigma-Aldrich) well-known for their antioxidant activity, were also tested in the same way.

Table 1. Percentages (mean \pm S.D.) of constituents found in St. John's Wort extracts (I to V).

Extract	Chlorogenic acid	Rutin	Hyperosid	Isoquercitrin	Quercitrin	Quercetin	I3,I18-biapigenin	Hypericin	Hyperforin
I	0.83 \pm 0.05	3.64 \pm 0.21	3.18 \pm 0.43	1.63 \pm 0.12	1.52 \pm 0.21	0.65 \pm 0.16	0.11 \pm 0.03	0.99 \pm 0.09	1.39 \pm 0.25
II	0.99 \pm 0.03	3.49 \pm 0.39	2.62 \pm 0.21	1.34 \pm 0.18	1.32 \pm 0.28	1.05 \pm 0.20	0.71 \pm 0.07	1.40 \pm 0.23	0.26 \pm 0.03
III	1.29 \pm 0.12	1.69 \pm 0.09	1.42 \pm 0.14	1.26 \pm 0.24	1.37 \pm 0.32	2.13 \pm 0.33	0.31 \pm 0.05	n.d.	n.d.
IV	1.15 \pm 0.15	0.17 \pm 0.02	0.43 \pm 0.08	0.44 \pm 0.03	0.41 \pm 0.11	0.22 \pm 0.03	0.04 \pm 0.01	n.d.	n.d.
V	1.28 \pm 0.07	2.15 \pm 0.32	1.33 \pm 0.11	0.96 \pm 0.09	0.75 \pm 0.16	0.14 \pm 0.08	0.04 \pm 0.01	n.d.	n.d.

n.d.: not detectable.

HPLC analysis

HPLC analysis was performed using the method by Brolis et al. (1998). The HPLC system consisted of a Waters instrument model 510 with a Waters 486 UV detector (Waters Milford, MA, USA). The reverse-phase column was a Polaris C-18 (150 \times 4.6 mm, 5 μ m) Varian (Varian Inc., Palo Alto, CA, USA), connected to a column prefilter Nova-Pak (C18, 4 μ m, 60 Å) (Waters), maintained at 30 °C. Chromatographic separation was carried out using three solvents [water: 85% phosphoric acid (99.7:0.3 v/v), acetonitrile, methanol], in a linear gradient programme at a flow rate of 1 ml/min. Peaks were detected at 270 nm.

The standards chlorogenic acid, rutin, hyperoside, isoquercitrin, quercetin, I3,I18-biapigenin were purchased from Extrasynthèse (Genay, France); hypericin and hyperforin from Sigma-Aldrich. Standard solutions were prepared in methanol HPLC grade and diluted to obtain calibration standards in the range 1 to 100 μ g/ml. Calibration curves were determined on four levels of concentration and calculated by linear regression analysis. All the curves had a coefficient of linear correlation \geq 0.998.

Extracts (I to V) were dissolved in water Milli-Q or methanol HPLC grade at concentration of 2.5 mg/ml. Each sample was then filtered through a cartridge-type sample filtration unit with Ultrafree Millipore and immediately injected. Analyses were performed at least in triplicate to calculate the mean percentage content of constituents.

Results were expressed as mean \pm standard deviation. Student's t-test was applied for detecting the significance of difference between different groups. P-values of 0.05 or less were considered significant. Differences between the means of two groups were evaluated by a two-tailed t-test for independent samples. Statistical analyses were

performed using the SPSS for Windows computer program (SPSS Inc., Headquarters, Chicago, IL, USA).

RESULTS AND DISCUSSION

The antioxidant activity of the five prepared extracts was measured *in vitro* with a spectrophotometric test. Each measurement was coupled with a HPLC analysis of the extract content in nine compounds (hypericin, hyperforin, chlorogenic acid, and the six flavonoids rutin, hyperoside, isoquercitrin, quercetin, quercitrin and I3,I18-biapigenin); all of which possess antioxidant properties and are usually considered representative of the various groups of molecules contained in St. John's wort (Brolis et al., 1998; Pietta et al., 2001; Avato and Guglielmi, 2004; Chandrasereka et al., 2005; Conforti et al., 2007; Sagrantini et al., 2008; Filippini et al., 2010) and showing this activity but different solubilities.

Data of the HPLC analysis of the various extracts are reported in Table 1. A comparison with previous analyses was not easy because of the more or less marked differences due to both extraction methods and the various biologic and environmental factors that are yet described affecting plant drug content. In general, it is however, possible to state that plant drug used in

the present study and collected on the Amiata Mountain showed a good/high content in hyperforin and in total flavonoids, and a good/medium content in hypericin. Besides the papers that are yet to be cited, it should be noted that the European Pharmacopoeia, in the specific monograph "St. John's Wort dry extract, quantified", requires hypericin 0.1 to 0.3%, total flavonoids minimum 6.0%, hyperforin maximum 6.0% (Ph.Eur., 2010).

It is also noteworthy that hydroalcoholic (with a low alcohol percentage) and aqueous extracts were fully devoid of hypericin and hyperforin. One should not wonder if these compounds are lacking. Hypericin is well known to be fully insoluble in water (Uzdensky et al., 2003), and many papers report methods to modify this molecule or to vehiculate it (Falk et al., 1998; Kubin et al., 2008). Also hyperforin is a relatively lipophilic compound and, furthermore, it is rapidly decomposed when exposed to heat or light (Römpp et al., 2004). An exhaustive study on the instability of St. John's wort constituents and degradation of hyperforin in aqueous solutions was published by Ang et al. (2004). As it concerns the other analyzed molecules, presence in different extracts followed extractive ability of used solvents and methods. Most compounds were better extracted by methanol, with the exception of chlorogenic

Table 2. IC₅₀ values of extracts (I to V).

Extract	IC ₅₀ values (µg/ml) mean ± S.D.	P value vs extract I
I	2.32 ± 0.16	
II	3.57 ± 0.20	0.01
III	5.24 ± 0.54	0.02
IV	7.87 ± 0.31	0.01
V	9.77 ± 0.56	0.01

Table 3. IC₅₀ values of some reference compounds.

Compound	IC ₅₀ (µg /ml)
Glutathione	3.69 ± 0.16
Captopril	5.43 ± 0.32
Rutin	3.85 ± 0.20
Quercetin	1.63 ± 0.10
Catechin	2.14 ± 0.18

acid which reached the maximum in extracts (III) and (IV).

Furthermore, between aqueous extracts (IV) and (V), it can be observed that all the present and analyzed constituents showed higher percentages in (V), obtained with boiling water, than in (IV), obtained at room temperature, the only exception being quercetin which however showed a low water solubility (Table 1).

All the extracts showed a good scavenging activity (IC₅₀ values in the range 2.32 to 9.77 µg/ml) decreasing from the most apolar extracts to the most hydrophilic ones (Table 2). Methanolic extract (I) exhibited an IC₅₀ value like the natural compound catechin (Table 3) and from HPLC analysis (Table 1) its most representative constituents were rutin and hyperosid. In extract (II) the pre-treatment with dichloromethane maintained the chromatographic profile, with the presence of all the looked for constituents, including hypericin and hyperforin, and a good scavenging activity, but lower than extract (I). The other extracts, obtained with more polar solvents, even if devoid of hypericin and hyperforin (which however contribute synergistically to the total antioxidant activity), showed IC₅₀ values higher in a statistically significant manner in respect of extract (I), but still very good if compared to reference compounds in Table 3.

Undoubtedly, on the basis of the percentages listed in Table 1, it is unclear why extract (V), is richer in analyzed compounds than extract (IV), and it showed a lower antioxidant activity. The only hypothesis is that molecules contributing to total antioxidant activity are very numerous, act synergistically and belong to different chemical groups (Kirmızıbekmez et al., 2009; Franklin et al., 2009). Although hot water has a higher extracting ability, a decoction lasting 15 min under reflux may promote degradation of many sensible constituents.

Conclusion

In conclusion, the different extracts, although prepared from the same plant drug in order to avoid all possible variations due to pedo-climatic factors, collection time and storage conditions, showed a very different phytochemical profile. This can contribute to explain the different therapeutical successes/failures and the dosage problems reported in recent literature on this plant drug. It is however, remarkable that extract (V), obtained by decoction, possessed a good antioxidant activity, with an IC₅₀ value still comparable with usual reference compounds. Considering also the amounts (cups) assumed by consumers, this means that home-made St. John's wort teas, although they are deprived of hypericin and hyperforin and it is discouraged to use them as antidepressant, it must be re-valued and reconsidered as possible beverages with good antioxidant properties.

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