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St. John's Wort (*Hypericum perforatum*) extracts and isolated phenolic compounds are effective antioxidants in several *in vitro* models of oxidative stress

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Abstract

The antioxidant potential of a total ethanolic extract of *Hypericum perforatum* (TE) was assessed, and correlated with the phenolic composition. TE was fully characterized by HPLC-DAD and HPLC-MSn, and phenolic compounds were isolated by semi-preparative HPLC. The free radical-scavenging properties of the TE were studied using DPPH$^{\cdot}$ ($EC_{50} = 49$ μg dwb/ml), AAPH ($EC_{50} = 50$ μg dwb/ml) and the antioxidant potential was evaluated using lipid peroxidation induced by ascorbate/iron ($EC_{50} = 28$ μg dwb/ml). Moreover, the TE was able to scavenge NO (by using sodium nitroprusside) and HOCl (by evaluating TNB reduction). Furthermore, the antioxidant potentials of the isolated compounds were evaluated and several compounds, namely flavonoids, were significantly less effective at DPPH$^{\cdot}$- and AAPH-scavenging, compared to their ability to prevent iron-mediated lipid peroxidation. Therefore, TE was able to scavenge several free radicals, indicating beneficial cellular protection against oxidative stress.

Keywords: *Hypericum perforatum*; Antioxidant activity; Radical-scavenging; Lipid peroxidation inhibition; Phenolics

1. Introduction

Oxidative stress has been considered an important factor in the onset and development of several chronic diseases. Natural antioxidants are interesting compounds due to their properties that help prevent oxidative stress (Youdim, Spencer, Schroeter, & Rice-Evans, 2002; Zheng & Wang, 2001), among other potentially beneficial actions. For instance, several biological effects have been attributed to flavonoids, such as antitumoral, anti-inflammatory, anti-ischemic and anti-aggregate plaquetary activities. These activities are believed to be in part related to the antioxidant properties of the compounds, namely in scavenging radical oxygen species (ROS) (Liu, 2004; Rice-Evans, 2001; Simonyi et al., 2005; Woodman & Chan, 2004).

Hypericum perforatum is a medicinal plant widely used, worldwide. The commercially available *H. perforatum*-derived products include sophisticated phytopharmaceuticals and nutraceuticals (Gaedcke, 2003). This plant has been indicated to have a wide range of applications. However, nowadays its use in the treatment of mild to moderate depression has become prominent (Butterweck, 2003; Caccia, 2005). *H. perforatum* medicinal properties have been associated with the phenolic composition. *H. perforatum* ethanolic extracts contain many polyphenolic compounds, namely flavonoids and phenolic acids, suggesting that they can be endowed with important antioxidant properties. Recently, we showed that *H. perforatum* extracts were highly effective in scavenging the stable free radical, DPPH$^{\cdot}$, and in inhibiting lipid peroxidation induced by ascorbate/iron in rat cortical synaptosomes (Silva, Ferreres, Malva, & Dias, 2005). Moreover, *H. perforatum* extracts were also efficient in protecting neurons

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from degeneration by exposure to the peptide β-amyloid (Silva, Dias, Ferreres, Malva, & Oliveira, 2004). The observed antiradicalar and antioxidant activities were related to the presence of hydroxycinnamic acids, namely, flavonoids. Other reports indicate that these extracts have a moderate oxygen radical absorbance capacity (ORAC) (Zheng & Wang, 2001) and significant scavenging capacity towards the superoxide radical produced by the xanthine/xanthine oxidase system (Hunt, Lester, Lester, & Tackett, 2001). However, available reports have not focused much attention on the type of ROS scavenged, or which compounds are effective, and a systematic structure-activity relationship is required.

The aim of this study was to evaluate the antioxidant properties of a total ethanolic extract of *H. perforatum* and of key isolated phenolic compounds, using several methodologies in order to establish a possible correlation between the observed antioxidant properties, the type of ROS, and the phenolic composition and structural characteristics of the isolated compounds.

2. Materials and methods

2.1. Chemicals

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), hypochlorous acid, sodium nitroprusside, sodium nitrite, sulfanilamide, *N*-1-napthylethylenediamine dihydrochloride (NED), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), trichloroacetic acid (TCA), sodium borohydride ($NaBH_4$), 2,2′-azo-bis(2-methylproprianamidine) dihydrochloride (AAPH), trolox, 1,1-diphenyl-2-picrylhydrazyl (DPPH·), BHT, and thiobarbituric acid (TBA) were purchased from Sigma (Barcelona, Spain). Sephadex LH-20 resin was supplied by Pharmacia (Uppsala, Sweden). Rutin, hyperoside, isoquercetin, quercetin, kaempferol, amentoflavone, apigenin, luteolin and hypericin were obtained from Extrasynthése (Genay, France). Hyperforin tetramonium salt was a gift of Dr. Clemans Erdelmeier (Wilmar Schwabe GmbH, Germany). Chlorogenic acid was purchased from Sigma (Barcelona, Spain). All other reagents were of analytical grade and supplied by Merck (Darmstadt, Germany).

2.2. Plant material and extracts of H. perforatum

H. perforatum plants (top parts) were collected in Braga (North of Portugal); the biomass was collected and freeze-dried on the same day. A single ethanolic extract batch was prepared by maceration of the biomass for one week, at room temperature, with an ethanol–water solution (80:20), final concentration of 100 mg dwb/ml. The mixture was filtered through a paper filter (Whatman, no. 1) and the resulting total ethanolic extract of *H. perforatum* (TE) was stored in a dark glass bottle for further processing.

2.3. Phenolic identification and quantification

TE was analysed by HPLC-DAD as described previously (Dias, Seabra, Andrade, & Ferreira, 1999). Briefly, analysis was performed using a Beckman System Gold chromatographic apparatus, composed of a quaternary pump (Model 126), a diode detector (Model 168), controlled by the PC-assisted System Gold Nouveau software. Chromatograms were recorded at 260, 350 and 590 nm. The quantification of the constituents was done by the external standard method, using a solution containing 15 μg/ml of each reference compound in methanol. The reference compounds were chlorogenic acid, rutin, hyperoside, isoquercitrin, quercetin, kaempferol, amentoflavone, hypericin and hyperforin salt. Other flavonols, flavones and caffeoylquinic acids were quantified at 350 nm, as quercetin, apigenin and chlorogenic acid equivalents, respectively. Hyperforins and hypericins were quantified at 260 and 590 nm as hyperforin and hypericin equivalents, respectively. All samples were analysed in triplicate.

Phenolic identification was also performed by HPLC-MSn as described elsewhere (Silva et al., 2005). Briefly, chromatographic separation was carried out on a RP C18 column (25 × 0.4 cm, particle size 5 μm, Merck, Germany), using water/formic acid (99:1) and methanol as the mobile phases. The HPLC system was an Agilent HPLC 1100 instrument series equipped with an Agilent DAD detector (G1315B, Agilent Technologies, Germany) and a ion-trap mass spectrometer (G2445A, Agilent Technologies, Germany) equipped with an electrospray ionization (ESI) system, in series. Mass scan (MS) and daughter (MS2) spectra were measured from 100 au to m/z 1500. Mass spectrometry data were acquired, in both negative and positive modes.

2.4. Compounds isolation

The TE was subjected to Sephadex LH-20 chromatography and semi-preparative HPLC as previously described (Dias, Tomás-Barberán, Ferreira, & Ferreres, 1998). Briefly, an aliquot of TE was evaporated under reduced pressure, at 35 °C in darkness. The TE residue was then solubilized in methanol and fractionated over Sephadex LH-20 (column: 40 × 3 cm), using methanol as the eluent. The fractions resulting from column elution were further analysed by TLC, grouped according to their chemical composition and evaporated separately. The fractions were re-dissolved in methanol and further separation was carried out on a RP C18 column (25 × 0.4 cm, particle size 5 μm, Merck, Germany), using water/formic acid (99:1) and methanol as mobile phases. Elution was performed using a gradient as described previously (Dias et al., 1998). This procedure was conducted in order to isolate the major phenolic compounds present in the TE. The isolated phenolics were identified by their UV/Vis characteristics and their mass spectra (Silva et al., 2005), and comparisons with pure standards.

2.5. Measurement of lipid peroxidation

The extent of lipid peroxidation was evaluated by measuring the levels of TBARS after exposure of rat (2 months old) cortical synaptosomes to ascorbate/iron. The synaptosomal fraction was isolated and the thiobarbituric acid assay was performed as described elsewhere (Silva et al., 2005). The synaptosomal fraction was diluted to obtain a final concentration of 0.5 mg protein/ml. Protein content was determined by the Biuret method, calibrated with bovine serum albumin. Lipid peroxidation was evaluated after incubation of synaptosomes with 800 μM ascorbic acid and 2.5 μM FeSO4, at 37 °C for 15 min, in the presence of *H. perforatum* extract or isolated compounds. Controls were made with synaptosomes, without the oxidant pair. BHT (0.45 mM, ethanol 100%) was used as a positive control. The absorbance of the (MDA)-TBA complex was detected at 530 nm. Values were corrected for the levels of basal peroxidation.

2.6. Measurement of antiradical activity

2.6.1. AAPH assay

The TE and isolated compounds were evaluated for their ability to inhibit oxidative stress induced by AAPH, an inductor of lipid peroxidation by formation of peroxyl radicals. This was assessed after incubation of synaptosomes with 75 mM AAPH, at 37 °C for 90 min. BHT (1 mM) was used as a positive control and the absorbance of the (MDA)-TBA complex was detected at 530 nm. Values were corrected for the levels of basal peroxidation.

2.6.2. DPPH· assay

The free radical-scavenger activity of the TE and isolated compounds was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay, as described previously (Silva et al., 2005). Briefly, the samples were prepared and diluted in ethanol and reacted with a solution of 80 μM DPPH·. The decrease in absorbance was monitored continuously at 517 nm with a Molecular Devices Spectra Max 340 PC, until the reaction was complete (plateau state). Ethanol was used as a blank and trolox (1 mM) was used as a positive control. All determinations were performed in triplicate. The percentage of reduced DPPH· at steady state was calculated and these values were plotted against the concentrations of individual fractions. The decrease in 50% of the initial DPPH· concentration was defined as the EC_{50}. All parameters were calculated graphically using the software GraphPad 4 (Prism, USA).

2.7. Evaluation of iron reduction and chelation

Ferrozine is a chromophoric chelator that strongly binds Fe^{2+}, forming a stable complex with a high extinction coefficient at 562 nm. Reduction of ferric iron was evaluated as described previously (Mira et al., 2002), with minor modifications. The index of reduction of ferric iron was calculated after incubation of a solution containing ferrozine (0.5 mM in sodium acetate buffer, pH 5.5) and $FeCl_3$ (15 mM) with several concentrations of TE. The amount of reduced iron was determined by the increase in absorbance (562 nm) after 5 min of reaction, being expressed as the ratio of Fe^{2+}/Fe^{3+}. Chelation of Fe^{2+} was evaluated using methodology similar to that of the previous assay. Briefly, the index of chelation of ferrous iron was calculated after incubation of a solution containing ferrozine (200 μM in sodium acetate buffer, pH 5.5) and $FeSO_4$ (70 μM). The amount of chelated iron was determined by the increase in absorbance (562 nm) after 10 min of reaction, and was expressed as the ratio of $Fe^{2+}_{chelated}/Fe^{2+}_{free}$.

2.8. Nitric oxide-scavenging

Sodium nitroprusside, a nitric oxide (NO) donor was used to assess the ability of TE to inhibit NO release, as described previously (Yokozawa & Chen, 2000). Sodium nitroprusside 5 mM in PBS was incubated with different concentrations of TE, at 37 °C for 2 h 30 min. Controls were made using ethanol (used to prepare the sample dilutions). The amount of NO released from sodium nitroprusside was assessed by measuring the accumulation of nitrite in the reaction mixture, using a microplate assay based on the Griess reaction.

2.9. Hypochlorous acid-scavenging – TNB oxidation

The ability of TE and isolated compounds to scavenge hypochlorous acid (HOCl) was evaluated as described previously (Valentão et al., 2002). Briefly, TNB was synthesized from DTNB (1 mM in 50 mM PBS containing 5 mM EDTA, pH 6.6) after addition of $NaBH_4$ (20 mM). The reaction of synthesis was performed at 37 °C for 30 min and the concentration of TNB produced was determined by measuring the absorbance at 412 nm, using the molar absorption coefficient of 13,600 $M^{-1}\,cm^{-1}$.

The solution containing TNB was diluted to obtain a concentration of 40 μM and reacted with a solution of 40 μM HOCl (prepared immediately before the assay by adjusting the pH of a stock solution of NaOCl to 6.2; concentration was determined spectrophotometrically at 235 nm using the molar coefficient of 100 $M^{-1}\,cm^{-1}$) and with several concentrations of the TE. The change in absorbance (412 nm) was monitored during the initial 10 min of reaction and expressed as a percentage of reduction.

2.10. Statistical analysis

All the measurements were made in triplicate, and at least two independent experimental sets were done. The data was analysed statistically with the Statistica/Mac software (Prism, USA). Mean differences were analysed statistically, running the one-way analysis of variance test (ANOVA). The homogeneity of variance was tested by Cochran's test.

3. Results

3.1. Phytochemical characterization of the total ethanolic extract (TE) and compounds isolated

The TE chromatogram profiles obtained were similar to those previously reported for *H. perforatum* ethanolic extracts (Silva et al., 2005). The main compounds were identified and quantified and the results are presented in Table 1.

All the compounds isolated had purities higher than 98%, as assessed by HPLC. The MSn data confirming the chemical structure of the isolated pure phenolic compounds are shown in Table 2.

Table 1
Phenolic composition of *H. perforatum* total ethanolic extract (TE)

Compounds	μg/g dwb	Compounds	μg/g dwb
Phloroglucinols		*Naphthodianthrones*	
Hyperforin	6880	Hypericin	570
Adhyperforin	1207	Pseudohypericin	699
Total	*8087*	Protopseudohypericin	48
		Protohypericin	27
		Total	*1334*
Flavonol glycosides		*Flavonoid aglycones*	
Hyperoside	7861	Quercetin	5128
Rutin	3949	Kaempferol	156
Isoquercitrin	1942	Biapigenin	2135
Querct 3-glucuronic + querct 3-galacturonic	352	Amentoflavone	108
Hyperoside-acetyl	758	*Total*	*7527*
Rutin-acetil	233		
Kampferol 3-rutinoside	283		
Quercetin 3-rhamnoside	3760		
Total	*19,138*		
Phenolic acids			
Neochlorogenic acid	2880		
Chlorogenic acid	1181		
Unidentified	189		
Total	*4250*		
		Total phenolics	*40,336*

(HPLC-DAD analysis).

Table 2
HPLC-MSn data of the major identified compounds of *H. perforatum*

Compound	$[M-H]^-$ (*m/z*)	$-MS^2[M-H]^-$ (*m/z*)
Rutin	609.0	300.4
Hyperoside	462.9	300.4
Isoquercetin	477.2	300.5
Quercitrin	593.5	284.4
Kaempferol 3-rutinoside	505.2	462.7, 300.2
Quercetin	302.6*	
Kaempferol	285.2	
Biapigenin	537.4	442.5, 384.7
Hypericin	503.3	405.3

* Ion obtained in the positive mode, corresponding to the protonation $[M+H]^+$.

The tested phenolics are indicated in Table 3, along with their structural features.

3.2. Antioxidant properties of the H. perforatum total extract (TE)

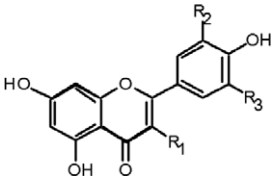
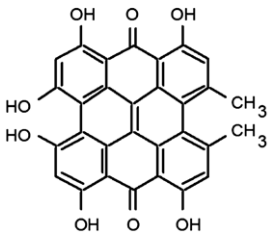
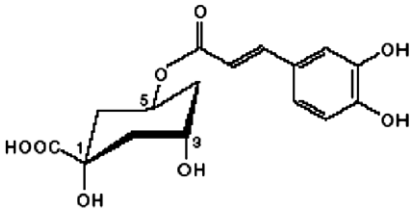
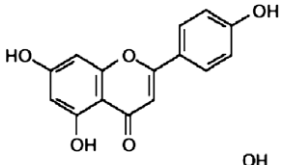
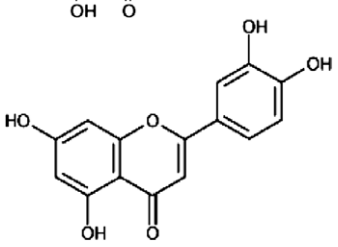
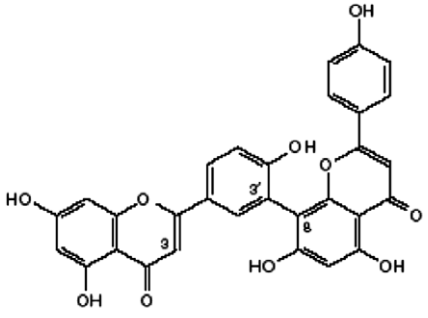
The free radical-scavenger activity of the TE was evaluated by reaction with either DPPH· or AAPH. The reduction of DPPH· absorbance is indicative of the capacity of the extracts to scavenge free radicals (or to donate electrons to the stable free radical DPPH·) (Vaya et al., 2003). AAPH is a water-soluble azo compound and a well known free radical initiator. The inhibition of lipid peroxidation induced by AAPH is an indicator of the ability of the extract and compounds to scavenge peroxyl radicals. The TE exhibited similar scavenging abilities towards DPPH· and AAPH (Table 4).

Additionally, we further characterized the antioxidant activity of the TE by indirectly evaluating its ability to scavenge NO and HOCl. Sodium nitroprusside is a compound that spontaneously releases nitric oxide (NO) in solution and the amount of NO released can be inferred by using the Griess reagent. This reagent reacts with nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO, and therefore allows an indirect estimation of the amount of NO released to the solution. The TE showed significant scavenging activity for 10 μg dwb/ml of TE ($78.7 \pm 1.3\%$ reduction of release) whereas, for a concentration ten times higher, the TE exhibited a decrease in potency ($58.2 \pm 2.6\%$ reduction of release). Results are shown in Table 5. Hypochlorous acid is a strong reactive oxygen species produced in organisms by oxidation of Cl^- ions at sites of inflammation (Valentão et al., 2002). HOCl induces the oxidation of TNB to DTNB. A HOCl scavenger inhibits this oxidation step, which can be monitored spectrometrically by changes in absorbance at 421 nm. As shown in Fig. 1, TE exhibited a moderate HOCl-scavenging activity, the maximal effect being attained for 50 μg dwb/ml TE with a reduction in TNB oxidation of $17.1 \pm 1.3\%$.

We further evaluated the extent of lipid peroxidation, after incubation, of rat cortical synaptosomes with the oxidant pair ascorbate/iron as another model of oxidative stress. MDA formation was used as an index of breakdown of membrane lipids. This is a useful method because it allows a rapid evaluation of the contribution of iron in mediating lipid peroxidation using biological membranes. Ascorbate maintains iron in a reduced state, thereby stimulating lipid peroxidation of the brain homogenate through various mechanisms, for example, through the decomposition of lipid peroxides, the generation of hydroxyl radicals, or by formation of perferryl or ferryl species (Ko, Cheng, Lin, & Teng, 1998).

Lipid peroxidation was significantly reduced in the presence of the TE (Table 6). The EC_{50}, determined for the TE inhibitory effect on lipid peroxidation, induced by ascorbate/iron, was 27.7 ± 1.3 μg dwb/ml. The marked ability

Table 3
Classes of phenolic compounds tested

	Structure	R1	R2	R3
Flavonoids				
Quercetin		OH	OH	H
Hyperoside		β-D-galactosyl	OH	H
Rutin		β-D-rutinosyl	OH	H
Isoquercitrin		β-D-glucosyl	OH	H
Quercitrin		α-L-rhamnosyl	OH	H
Kaempferol		OH	H	H
Kaempferol-3-rutinoside		O-β-D-glc-(6<-1)α-L-rha	H	H
Naphthodianthrones				
Hypericin				
Phenolic acids				
Chlorogenic acid*				
Flavones				
Apigenin*				
Luteolin*				
Biflavones				
Biapigenin Amentoflavone*		I3, II8-biapigenin I3′, II8-biapigenin		

Compounds with an asterisk (*) were not isolated from TE but were purchased.

Table 4
Antiradical potential of *H. perforatum* total ethanolic extract (TE) and isolated phenolic compounds

	EC_{50}	
	DPPH·	AAPH
Trolox	20.8 ± 1.03	n.t.
BHT	n.t.	97.5 ± 0.71
TE	49.3 ± 1.05	50.4 ± 2.57
Quercetin	8.30 ± 1.03 a, b	29.4 ± 2.29 a, b**
Hyperoside	6.38 ± 1.06 a	11.5 ± 1.76
Rutin	11.3 ± 1.06 b, c	31.5 ± 4.93 a*
Isoquercitrin	11.7 ± 1.02 c	n.d.***
Quercitrin	13.0 ± 1.10 c	n.d.***
Kaempferol	21.3 ± 1.04 d	n.d.***
Kaempferol-3-rutinoside	n.d.	n.d.
Chlorogenic acid	20.3 ± 1.04 d	22.6 ± 1.19 b
Luteolin	15.8 ± 1.04	n.t.***
Apigenin	n.d.	n.d.
Biapigenin	n.d.	n.d.
Amentoflavone	n.d.	n.d.
Hypericin	n.d.	n.d.

Antiradical potential was evaluated by using the stable DPPH radical in solution, and by evaluating the scavenging of peroxyl radicals formed in lipid membranes by AAPH.The values are presented as means ± SEM of three independent experiments. The EC_{50} values are expressed as μg dwb/ml (TE) and as μM (pure compounds). In each column, values are statistically different ($p < 0.05$), except those marked with the same letter. In each line all the values are similar, except those marked with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$); n.d. – scavenging activity below 50% for the maximum concentration tested (75 μM).

Table 5
Inhibition of nitric oxide release by *H. perforatum* total ethanolic extract (TE)

TE (ug dwb/ml)	Reduction NO release (%)
10	78.7 ± 1.30
100	58.2 ± 2.55

The values are presented as means ± SEM of three independent experiments and are significantly different ($p < 0.05$).

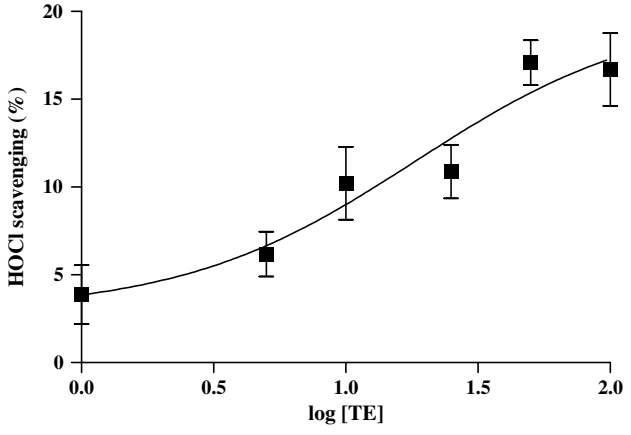


Fig. 1. Hypochlorous acid (HOCl)-scavenging by *H. perforatum* total ethanolic extract (TE). Activity was measured by reduction of the TNB to DTNB, by scavenging of HOCl. The values are presented as means ± SEM of three independent experiments.

Table 6
Lipid peroxidation inhibition potential of *H. perforatum* total ethanolic extract (TE) and isolated phenolics

	Ascorbate/iron (EC_{50})
TE	27.7 ± 1.26
Quercetin	0.08 ± 1.90 a
Hyperoside	5.37 ± 1.05 b, c
Rutin	8.98 ± 1.03 b
Isoquercitrin	5.79 ± 1.05 b, c
Quercitrin	7.33 ± 1.16 b
Kaempferol	0.69 ± 1.62 a, c, d
Kaempferol-3-rutinoside	n.d.
Chlorogenic acid	39.2 ± 3.12
Luteolin	0.75 ± 1.08
Apigenin	5.19 ± 1.96 b, d
Biapigenin	5.10 ± 1.11 b, d
Amentoflavone	4.53 ± 1.09 b, d
Hypericin	21.0 ± 2.86

The values are presented as means ± SEM of three independent experiments. The EC_{50} values are expressed as μg dwb/ml (TE) and as μM (pure compounds). n.d. – scavenging activity below 50% for the maximum concentration tested (75 μM). Values are statistically different ($p < 0.05$), except those marked with the same letter.

of the TE to inhibit iron-mediated lipid peroxidation could also, in part be, due to interactions with iron. Therefore, we evaluated the ability of the TE to reduce and chelate iron. The reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), and chelation of iron were evaluated in the presence of ferrozine, by the production of a chromophore that absorbs at 562 nm, and several concentrations of the TE (Table 7). The TE was able to reduce Fe^{3+} and chelate Fe^{2+} in a concentration-dependent way. Linear relations were obtained, both for reduction and chelation. The slopes calculated were 0.006 nmol Fe^{3+}/μg dwb TE ($r^2 = 0.9844$; for reduction) and 0.044 nmol Fe^{2+}/μg dwb TE ($r^2 = 0.9987$; for chelation). Ascorbate and EDTA were used as controls for reduction and chelation, respectively.

3.3. Antioxidant properties of isolated compounds

The more pronounced activity of the TE towards the DPPH· and AAPH radicals, and inhibition of iron-medi-

Table 7
Reduction (ferric iron, Fe^{3+}) and chelation properties (ferrous iron, Fe^{2+}) of HP total ethanolic extract (TE), analysed by abstraction of Fe^{2+} by ferrozine

TE (μg dwb/ml)	Reduction ($Fe^{3+}_{red}/Fe^{3+}_{free}$)	Chelation ($Fe^{2+}_{chel}/Fe^{2+}_{free}$)
5	n.d.	0.17
10	1.03	0.22
20	1.10	n.d.
25	n.t.	0.94
50	1.35	1.99
100	1.60	n.d.
Slope	0.006 ($r^2 = 0.984$)	0.044 ($r^2 = 0.999$)

The values are presented as means of three independent experiments. The concentrations for TE are expressed as μg dwb/ml. n.d. – not detected. Ascorbate = 50 mM (54.5% reduction); EDTA = 100 mM (100% chelation).

ated lipid peroxidation, comparatively to the other antioxidant models tested, led us to clarify which of the compounds present in the TE could contribute most significantly to the observed activities. Therefore, we studied the free radical-scavenging properties and lipid peroxidation inhibition of the isolated phenolics in these *in vitro* models.

Most of the compounds tested, were highly efficient in scavenging DPPH$^{\cdot}$ as shown by their EC_{50} values, with the exception of the flavones and hypericin (Table 4). These compounds were not able to reduce 50% of the DPPH$^{\cdot}$ present in the reaction solution for the highest concentration tested (75 μM). However, luteolin, a flavone used as a reference, was able to reduce DPPH$^{\cdot}$ ($EC_{50} = 15.8 \pm 1.0$ μM). The most effective compounds for scavenging DPPH$^{\cdot}$ were hyperoside and quercetin with EC_{50} values of 6.4 ± 1.1 and 8.3 ± 1.0 μM, respectively. AAPH scavenging was significantly less pronounced than DPPH$^{\cdot}$ scavenging and several compounds were not effective (Table 4). Hyperoside was the most effective AAPH scavenger ($EC_{50} = 11.5 \pm 1.7$ μM).

Lipid peroxidation was significantly reduced in the presence of the tested compounds, with the exception for kaempferol-3-rutinoside. For this compound, lipid peroxidation was only reduced by 10% for the maximum concentration tested (75 μM). Among the compounds present in TE, the most effective for reducing lipid peroxidation were quercetin and kaempferol (0.1 ± 1.9 and 0.7 ± 1.6 μM, respectively). It is noteworthy that the flavonoid aglycones were, overall, more effective than were the respective glycosides. Results are shown in Table 6.

4. Discussion

It has been proposed that therapies targeting oxidative aggressions in pathological conditions involving oxidative stress, are beneficial. Recently, some attention has been focused on the antioxidant properties of *H. perforatum*. Hunt et al. (2001) reported the ability of *H. perforatum* extracts to scavenge superoxide radical. More recently, we and others have reported that extracts from *H. perforatum* are also effective scavengers of the free stable DPPH radical (Benedi, Arroyo, Romero, Martin-Aragon, & Villar, 2004; Silva et al., 2005). Particularly, TE fractions containing flavonoids were very effective (Silva et al., 2005).

In the present study, the observed antiradicalar potency of the TE was considerably inferior to its capacity to inhibit iron-mediated lipid peroxidation (Tables 4 and 6). These differences may be due to the phenolic composition of the extract: the TE has several aglycones that could effectively scavenge ROS in lipidic environments, accounting for the observed higher antioxidant activity. However, when individually tested, most of the compounds were poor peroxyl radical-scavengers, suggesting that the differences in the ability to scavenge free radicals in aqueous or lipidic media might not be the only causative factor for the observed differences in the antioxidant activities of the TE.

The TE was able to reduce a considerable amount of iron and to chelate approximately 50% of the total iron present in solution, for concentrations similar to the EC_{50} values estimated for the lipid peroxidation assay. Therefore, the marked differences between the antiradical activity and inhibition of lipid peroxidation are more likely due to the presence of compounds possessing antioxidant properties together with iron-binding ability. It is well established that several phenolics are effective iron chelators (Brown, Khodr, Hider, & Rice-Evans, 1998; Mira et al., 2002). Flavonoids are a class of phenolic compounds present in high amounts in TE and reported to be strong antioxidants, able to form stable non-prooxidant complexes with iron (Rice-Evans, 2001).

It was previously reported that flavonoids played a major role in antioxidant activity (Arora, Nair, & Strasburg, 1998). The catechol structure together with the 4-oxo-3-hydroxyl moieties, were regarded as relevant for metal chelation by flavonoids (Brown et al., 1998; Engelmann, Hutcheson, & Cheng, 2005). Among the compounds tested, quercetin, kaempferol and luteolin were the most effective in preventing lipid peroxidation (Table 6). The conjugation of both the catechol and the 4-oxo-3-hydroxyl groups are relevant for the antioxidant properties and these accounted for the higher activity of quercetin, than kaempferol or luteolin. The presence of the 4-oxo-3-hydroxyl in kaempferol accounted for a higher activity than luteolin, and the presence of the catechol group would account for the higher efficacy of luteolin than of apigenin.

To a lesser extent, all quercetin-glycosylated derivatives were effective inhibitors of iron-mediated lipid peroxidation. Some authors have described that the glycosylation in the three-position could mask the antioxidant activity, by decreasing the binding affinity for iron. Additionally, glycosylation also alters the lipophilicity and the bulkiness of the residue preventing the compound from being completely conjugated, which would be reflected in the binding of the compounds in lipid membranes and, consequently, in their lipid peroxidation-inhibiting efficacy (Cos et al., 2001). Hyperoside was an exception to the general concept that glycosylation masks or decreases the ability of a flavonoid, as an efficient inhibitor of lipid peroxidation, and also an effective radical-scavenger. This compound showed interesting properties, such as antidepressive activity (Butterweck, 2003). Kaempferol-3-rutinoside was ineffective in protecting against iron-mediated lipid peroxidation. The lack of the catechol group, and the substitution at position 3-OH by a bulky 3-*O*-rutinosyl group, could explain the low efficacy of this compound compared to kaempferol. A similar behaviour was observed for rutin, that showed the lowest values for both inhibition of lipid peroxidation and peroxyl radical-scavenging, compared to the other quercetin-glycosylated derivatives tested.

Among the aglycones tested, apigenin, amentoflavone and biapigenin inhibited lipid peroxidation, with similarly potency to flavonoid glycosides, despite being very poor radical-scavengers (Tables 4 and 6). A possible contribution

from iron chelation could explain the inhibition of lipid peroxidation observed for these compounds. van Acker, van Balen, van den Berg, Bast, and van der Vijgh (1998) reported that apigenin was able to chelate iron (van Acker et al., 1998), and, possibly, that the iron-binding properties are retained to some extent in the biflavones, amentoflavone and biapigenin.

Among the flavonoids, quercetin (and its glycosyl derivatives) and luteolin were efficient radical-scavengers (Table 4), particularly DPPH•, which supports the relevance of the catechol group for radical-scavenging properties (Silva et al., 2002).

Hyperoside and chlorogenic acid were the most effective peroxyl radical-scavengers. With the exception of quercetin and rutin, none of the remaining compounds tested showed significant activity. It has been previously reported that a flavonoid extract of *H. perforatum*, enriched in hyperoside, rutin and quercetin showed peroxyl-scavenging activity (Zou, Lu, & Wei, 2004). Laranjinha, Almeida, and Madeira (1994) reported chlorogenic acid to be an effective scavenger against peroxyl radicals (Laranjinha et al., 1994). Chlorogenic acid has been reported to inhibit lipid peroxidation by scavenging peroxyl radicals, thereby preventing the initiation of chain lipid peroxidation (Kono et al., 1997). However, in our study the lower efficacy of chlorogenic acid in preventing lipid peroxidation, when compared to flavonoids, is likely due to the presence of only one site for iron chelation in the molecular structure of chlorogenic acid (Kono et al., 1998).

Oxidative stress has been implicated in exacerbated inflammation, a process of cellular aggression mainly mediated by reactive oxygen/nitrogen species (ROS/RNS). Our results suggest that TE could have a beneficial role in inflammatory disorders by trapping two important mediators of inflammatory processes: nitric oxide (NO) and hypochlorous acid (HOCl). Previous studies have reported that compounds also present in *H. perforatum* extracts, such as chlorogenic acid and flavonoids, are efficient scavengers of HOCl (Firuzi, Mladenka, Petrucci, Marrosu, & Saso, 2004; Kono et al., 1998).

Taken together, the results indicate that the TE has several compounds with antioxidant properties, able to scavenge several types of ROS. These might explain the popular use of TE extracts for several distinct pathologies involving oxidative stress, e.g. skin wounds, eczema, burns, inflammation, and psychological disorders. Furthermore, the combination of metal-chelating properties and free radical-scavenging abilities of TE and other compounds present might be highly relevant for their utilization as phytopharmaceuticals in some CNS disorders. The data obtained from the present work further support the idea that the consumption of TE as an ingredient in functional foods or as a nutraceutical might have a beneficial effect for health, due to its multiple antioxidant properties and low side effects. Nevertheless, it should also be emphasised that some TE-drug interactions occur (Schulz & Johne, 2005), demanding a better knowledge of the effective dosages of these type of products, under which no adverse effects are found.

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