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Full Length Research Paper

Antioxidant properties, total phenolic, total carotenoid and chlorophyll content of anatomical parts of *Hypericum foliosum*

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Methanolic extracts from eight anatomical parts of the endemic Azorean *Hypericum foliosum* were evaluated for their potential antioxidant activity and basic classes of bioactive compounds. The results revealed that one single method is not enough to perform an accurate estimation of the antioxidant potential. The reducing power, prevention of β-carotene/linoleic acid system oxidation and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities are very promising in comparison with reference compounds, while none of the extracts showed significant iron-chelating ability. On the other hand, a pro-oxidant action was observed for six of the extracts regarding the inhibition of protein oxidative damage. Among all assayed samples, the stems showed significant antioxidant properties, a fact which may be related to the high total phenolic content (386.89 mg of gallic acid equivalents/g dry extract), which is twice than the majority of the other samples. The present data on *H. foliosum* revealed a potential source of powerful antioxidants, for further detailed phytochemical investigation.

Key words: *Hypericum foliosum* Aiton, total phenolic content, DPPH radical scavenging activity, β-carotene/linoleic acid assay, reducing power assay, superoxide anion activity, metal chelating activity, BSA oxidative damage assay.

INTRODUCTION

It is well known that the reactive oxygen species play an important role in the development of various serious diseases, such as neurodegenerative disorders, cancer, arteriosclerosis and rheumatism (Halliwell, 1999). To compensate this deleterious effect, the WHO recommends the use of natural antioxidants that can delay or inhibit the lipids or other molecules oxidation by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998).

A great number of aromatic, spicy, medicinal (example, rosemary, grape, oregano, green tea) and other plants containing chemical compounds exhibiting antioxidant properties were the target of several studies which resulted in the development of natural antioxidant formulations for food, cosmetic and other applications. However, scientific information on antioxidant properties of endemic plants, limited to certain regions and known only by local populations, is still rather scarce. Therefore, the assessment of such properties remains an interesting and useful task, particularly to find new promising sources of natural antioxidants for functional foods and/or nutraceuticals (Balasundram et al., 2006; Miliauskas et al., 2004).

Plant species of the genus *Hypericum* (*ca* 450 species) are well known for its use in folk medicine, due to the therapeutic efficacy of its many different species (Robson, 2003). Their health benefits are demonstrated

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through a number of pharmacological and clinical trials (Barnes et al., 2001; Greeson et al., 2001), specially in the most important and recognized species of the genus, *Hypericum perforatum* (St. John's Wort), which has been used in herbal medicine, externally for the treatment of skin wounds, eczema and burns and, internally, for disorders of the central nervous system, the alimentary tract and other purposes (Barnes et al., 2001; Bombardelli and Morazzoni, 1995).

A review of the recent literature shows that data on the antioxidant activity of *Hypericum* species is rather scarce or limited to a reduced number of species and its varieties. Besides, the correlation between this property and the phenolic composition is not always investigated in many studies. Some researchers established that caffeoylquinic acids (chlorogenic acid) and flavonoids, particularly quercetin and its glycoside derivatives, are the main compounds responsible for the antioxidant properties of St. John's Wort aqueous and organic extracts (Silva et al., 2005; Silva et al., 2008). These classes of compounds possess a broad spectrum of biological activities including radical scavenging properties (Balasundram et al., 2006; Havsteen, 2002), that could be, in part, responsible for some of the medicinal claims that are attributed to this plant, namely those related to free radical generation, such as the anti-inflammatory properties (Silva et al., 2005). Despite detailed studies on the aerial parts of some *Hypericum* species, information on the antioxidant activity and composition of different morphological parts is still scarce.

H. foliosum Aiton (HF) is an endemic species of the Azores archipelago (Portugal) classified under the section *Androsaemum* characterized by the absence of dark glands (Franco, 1971). To the best of our knowledge, no studies on the use of this plant in folk medicine have been reported so far, although local population describes its therapeutical properties as similar to those known for other *Hypericum* species (oral information). Only two phytochemical reports appeared on this species, one dealing with the essential oil characterization (Santos et al., 1999) and other with the isolation, from the aerial parts, of a new acylpholoroglucinol derivative with anti-staphylococcal activity (Gibbons et al., 2005). In a previous study (Rainha et al., 2011) our research group found that the aqueous extracts of aerial parts of HF presents high radical scavenging activity and higher levels of phenolic compounds as compared with St. John's Wort and other two common species of *Hypericum* in the Portuguese market, namely, *H. androsaemum* and *Hypericum undulatum*.

Bearing all this in mind, in this study, eight different anatomical parts of HF were extracted with methanol and analyzed for its antioxidant properties, total phenolics, total carotenoids and chlorophyll content employing commonly accepted assays. The antioxidant activity was extensively evaluated using six different established protocols and its potential correlation with the chemical composition of the extracts was investigated.

MATERIALS AND METHODS

Chemicals

Butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid (LA), Tween 20, gallic acid, sodium carbonate, Folin-Ciocalteu reagent (2N), bovine serum albumin (BSA), nitro blue tetrazoliumchloride (NBT), β-nicotinamide adenine dinucleotide (β-NADH), ferrozine, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), ethylenediamine tetraacetic acid (EDTA), hydrochloric acid (37%, p.a.), ferric chloride, iron (II) chloride tetrahydrate, potassium ferricyanide, citric acid (99.5%) and guanidine hydrochloride were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol, ethanol, ethyl acetate, ammonium acetate, sodium dihydrogen phosphate, glacial acetic acid, 5-methylphenazinium methosulphate (PMS), 2, 4-dinitrophenylhydrazine (2, 4-DNPH), hydrogen peroxide and chloroform were purchased from E. Merck (Darmstad, Germany). Deionised water was obtained from an in-house Milli-Q water purification system (Millipore, Bedford, MA, USA).

Plant material and extraction

A fully developed HF plant (1.5 m high) was collected in April 2009 at São Miguel Island (650 m, Pico da Cruz, Vila Franca do Campo region). The plant was washed, cleaned and separated in eight different anatomical parts: aerial parts (AP), young leaves (YL), old leaves (OL), stem bark (SB), stems (ST), root (RO) and seed capsules (SC). Flowers (FL) were collected in July 2009 in the same region during the flowering period. The plant material, after withering at ambient temperature in the dark airy rooms until they moisture reach 5%, was stored and protected from light and humidity until further chemical analysis. A voucher specimen (voucher number UA-DCTD-162) was deposited at the Department of Technological Sciences and Development, University of Azores.

The extraction procedure was based on the method described by Pellati et al. (2005). 1 g of finely powdered plant material was extracted with 50 ml of methanol at room temperature using a magnetic stirring bar for 30 min. The extracts were filtered through a type HA 0.45 µm membrane filter (Millipore, Bedford, MA, USA), concentrated to dryness using a rotary evaporator under reduced pressure at 30ºC and stored at -20ºC until further analysis. As far as possible, all extraction procedures were performed under daylight protection.

Total phenolics, total carotenoids and chlorophyll content

Determination of total phenolic content (TPC)

TPC in plant methanolic extracts was determined by using Folin-Ciocalteu colorimetric methodology based on the oxidation/reduction reaction as described by Waterhouse (2002). Determinations were carried out in triplicate averaged and calculated from a calibration standard curve of gallic acid. An aliquot of 20 µL of each sample solution and each calibration solution or blank was pipetted into separated cuvettes and 1.58 ml water plus 100 µl of 2 N Folin-Ciocalteu reagent were added and

well mixed. Additionally, 300 µl of 20% (w/v) Na_2CO_3 was added within 30 s to 8 min interval and shake to mix. The sample was incubated for 2 h at room temperature. The absorbance (Abs) was measured at 765 nm in a Heλios α UV/VIS spectrophotometer and was plotted against concentration using methanol as blank. TPC in plant extracts was expressed in gallic acid equivalents (GAE) and was calculated using the following equation:

$C = cV/m$

where C is the total content of phenolic compounds, mg GAE/g dry extract (dE); c the concentration of gallic acid obtained from the calibration curve, mg/ml; V the volume of extract, ml and m is the weight of extract, g.

Determination of total carotenoids and chlorophylls a and b

Methanolic solutions of plant extracts of the appropriate concentration (1.0 to 4.0 mg/ml) were analyzed in a Heλios α UV/VIS spectrophotometer at 470, 653 and 666 nm. The concentrations of carotenoids and chlorophylls α and b were determined according to the equations reported by Lichtenthaler and Wellburn (1985) as follows:

Total carotenoids (mg/L) = $1000\ Abs_{470} - 2.860\ C_a - 129.2\ C_b/245$

Chlorophyll α (mg/L) = $15.65\ Abs_{666} - 7.340\ Abs_{653}$

Chlorophyll b (mg/L) = $27.05\ Abs_{653} - 11.21\ Abs_{666}$

Determination of antioxidant activity

Free radical scavenging activity (FRSA) assay

The FRSA of plant extracts were determined in a methanolic solution of DPPH, used as a stable radical, according to the method described by Molyneux (2004). An aliquot of 1 ml of the methanolic sample solution or BHT (33.3 µg/ml) was placed in a test tube and 2 ml of 20 µM DPPH methanolic solution was added. Methanol was used as a blank to zero the Abs, BHT was used as reference sample and mixture without plant extract or BHT was used as the control (c). The Abs was measured at 517 nm over a period of 30 min and after each 5 min of reaction time. The FRSA of the sample (s) was calculated as a percentage of DPPH decolouration using the following equation:

$\%FRSA = (1 - Abs_s/Abs_c) \times 100$

β-Carotene/LA assay

For the β-carotene/LA assay, the method described by Dapkevicius et al. (1998) was used with some modifications. A stock solution of β-carotene and LA was prepared as follows: 0.5 mg of β-carotene was dissolved in 1 ml of chloroform, and 25 µl of LA plus 200 mg of Tween 20 were added. After chloroform evaporation under vacuum, 100 ml of distilled water was added to the residue. An aliquot of 500 µl of each plant extract or BHT (90 µg/ml) was pipetted into separated test tubes and 5 ml of the previous mixture was added. The test tubes were incubated for 2 h at 50 ºC together with the control sample. The Abs was measured at 470 nm at the beginning (t = 0 min) and after the experiment (t = 120 min). All determinations were performed in triplicate and averaged. The antioxidant activity (AA) was calculated as percentage inhibition of oxidation using the following equation:

$\%AA = [1 - (Abs_s^{0} - Abs_s^{120})/(Abs_c^{0} - Abs_c^{120})] \times 100$

Reducing power assay

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Each extract (concentration range 15.6 to 62.5 µg/ml) in methanol (0.4 ml) was mixed with 0.4 ml of 300 mM of phosphate buffer (pH 6.6) and 0.4 ml of potassium ferricyanide (1%, w/v), in a 2 ml eppendorf, and the mixture was incubated at 50ºC for 20 min. After 0.4 ml of TCA (10%, w/v) was added, the mixture was centrifuged at 10000 rpm for 5 min. The upper layer (1 ml) was mixed with 1 ml of deionised water and 0.2 ml of $FeCl_3$ (0.1% w/v), and the Abs was measured at 700 nm against a blank. The blank solution contained pure methanol instead of the methanolic plant extract. A higher Abs indicates a higher reducing power. EC_{50} value (µg extract/ml) is the effective concentration at which the Abs was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. BHT was used for comparison.

Superoxide anion scavenging activity assay

Measurement of superoxide anion scavenging activity was based on the method described by Liu et al. (1997) with slight modification. Superoxide anion radicals were generated in a PMS-NADH system by oxidation of NADH and assayed by reduction of NBT. In this experiment, the superoxide anion radicals were produced in 2 ml of phosphate buffer (100 mM, pH 7.4) containing 78 µM NADH, 50 µM NBT and samples at different concentrations (concentration range 31.3 to 125.0 µg/ml). After 5 min incubation at room temperature, PMS (10 µM) was added to initiate the reaction and after another 5 min incubation at room temperature the colour reaction between superoxide anion radical and NBT was read at 560 nm. Mixture without sample was used as control and mixture without PMS used as blank. Gallic acid was used for comparison. The scavenging activity was calculated as follows:

$\%Scavenging\ activity = [(Abs_c - Abs_s)/Abs_c] \times 100$

Ferrous ion-chelating assay

Chelating ability was determined according to the method of Dinis et al. (1994). An aliquot of 1 ml of each methanolic extract (concentration range 50 to 200 µg/ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the Abs was determined at 562 nm. Methanol instead of sample solution was used as a control. Methanol (0.2 ml) instead of ferrozine solution was used as a sample blank (b), which is used for error correction because of unequal colour of the sample solutions. EDTA was used as reference standard. A lower Abs indicated a better ferrous ion-chelating ability. The ferrous ion-chelating ability was calculated as follows:

$\%Ferrous\ ion\text{-}chelating\ ability = [1- (Abs_s\text{-}Abs_b)/Abs_c] \times 100$

BSA oxidative damage assay

The Makris and Rossiter (2001) system based on Fenton-reaction-mediated BSA modification was used to evaluate the efficiency of methanolic extracts to inhibit protein oxidation. Oxidation of BSA was performed in phosphate buffer (pH 7.4). 50 µl of BSA (20.0 mg/ml), 50 µl of $FeCl_2$/citric acid (4.0/4.0 mM), 50 µl of H_2O_2 (4.0

mM) and 50 µl of a sample solution (concentration range 62.5 to 250 µg/ml) were placed in a 1.5 ml eppendorf tube. The mixture was incubated in a water bath at 37°C for 60 min.

Carbonyl content of oxidized BSA was determined according to a previously published method of Levine et al. (1990). 0.5 ml of 2,4-DNPH (10 mM in 2 N HCl) was added to the samples and the resulting mixture was allowed to react for 60 min at room temperature, with vortexing every 15 min. After the completion of the reaction, 0.5 ml of TCA (20%) was added to precipitate protein, samples were centrifuged for 5 min, and the supernatant was discarded. The precipitate was washed three times with 1 ml of EtOH/EtOAc (1:1), each time centrifuging and discarding the supernatant. The washed precipitate was dissolved in 0.6 ml of guanidine (6 M in phosphate buffer, adjusted to pH 2.3 with TFA), incubated at 37°C for 15 to 20 min and centrifuged. Results are expressed as % inhibition in carbonyl formation, relative to control, from the Abs at 390 nm. BHT was used for comparison.

Statistical analysis

All the measurements were made in triplicate, mean centered and scaled to equal unit variance prior to be analysed statistically running the one-way analysis of variance test (ANOVA) using the XLSTAT 2010 statistical package for Windows. Mean differences were determined by Duncan's test for separation of means showing significant differences at $P < 0.05$. Significant correlations between all the assays were achieved by Pearson correlation coefficient (r) at a significance level of 95% ($P< 0.05$). The antioxidant properties, total phenolic and total carotenoids content data obtained for the different samples were statistically processed using hierarchical agglomerative cluster analysis by unweighted pair-group average. Similarity was measured by the Pearson correlation coefficient and the results visualized by a dendrogram.

RESULTS AND DISCUSSION

Total phenolics, total carotenoids and chlorophyll content

The TPC in the methanolic extracts was calculated from regression equation of calibration curve ($y = 1.0091x – 0.0141$; $R^2 = 0.998$) and was expressed as mg of GAE per g of dE. The TPC values of the different parts of HF ranged from 37.47 to 386.89 mg GAE/g dE, as shown in Table 1, being the TPC from the ST extract at least about twice higher than the other samples. The YL and OL extracts have shown no significant difference (139.25 and 146.25 mg GAE/g dE, respectively). The AP, RO, SB and FL presented statistically related values between 174.13 and 205.65 mg GAE/g dE. From these results it is clear that in all extracts under study, with the exception of the SC extract, phenolics are in abundance.

Methanol is a very good solvent for chlorophylls extraction, particularly from vascular plants and algae (Porra et al., 1989). The highest total quantities were observed in the AP, YL and OL extracts (Table 1), as expected. The ST and SB extracts presented also chlorophylls a and b. The RO, SC and FL extracts did not present these pigments. Despite their potential antioxidant capacity especially towards the lipid peroxidation mechanism (Lanfer-Marquez et al., 2005), these pigments are minor constituents in these samples and probably do not contribute significantly to the overall antioxidant activity.

Carotenoids play an important role in plant reproduction: along with phenolics they are responsible for bright colours. This chemical class act as antioxidants, with functions that include protection of membranes against damage by free radicals and retardation of ageing processes (Bulda et al., 2008). Table 1 show the spectrophotometrical measurement of total carotenoids based at the sample Abs at 470 nm. The results revealed that the AP, ST and SB extracts are the most rich in these components. The YL and OL extracts presented very similar quantities of total carotenoids. The RO, SC and FL extracts presented the lowest values. The amount of total carotenoids in these samples is significant and along with total phenolics represents a big portion of the extracts.

The determination of the phenolic profiles of the methanolic extracts of different anatomical parts of HF is presently in progress. The main compounds present in the AP extract are caffeoylquinic acids (chlorogenic acid), quercetin 3-sulphate and a major lipofilic compound (not identified yet). The differences in the chemical profile of the YL, OL and FL extracts are mostly quantitative as compared with AP extract. The RO, ST and SB extracts does not present quercetin 3-sulphate, being the chemical composition consistent to the other plant parts. SC has a unique chemical composition since none of the mentioned compounds was detected.

Antioxidant activity

Natural antioxidants are considered to be multifunctional and of high interest as alternatives to synthetic antioxidants to prevent oxidation in complex food systems (Wang et al., 2009). Their activity depends on various parameters such as the multiplicity and heterogeneity of the matrix, the experimental conditions and mainly the reaction mechanism. It is now known that the antioxidant properties of plant extracts cannot be evaluated by one single method due to the complex nature of phytochemicals. A thorough antioxidant assay of plant extracts should involve several activity studies (Gioti et al., 2009). Bearing this in mind, the antioxidant properties of methanolic extracts of eight anatomical parts of HF was evaluated by six different tests and was compared with that from well known antioxidants.

FRSA

The FRSA measure the hydrogen atom or electron donor capacity of an extract to the stable radical DPPH formed in solution. In other words, it measures the capacity of

Table 1. Total phenolics, total carotenoids and content of chlorophylls a and b of methanolic extracts of *Hypericum foliosum* anatomical parts*.

Sample	Ref.	Total phenolics (mg GAE/g dE)	Total carotenoids (mg/g dE)	Chlorophyll α (mg/g dE)	Chlorophyll b (mg/g dE)
Aerial parts	AP	174.13±2.99 a	99.57±3.96 a	1.50±0.12 a	0.54±0.06 a
Young leaves	YL	139.25±3.35 b	49.75±2.21 b	0.79±0.09 c	0.36±0.05 c
Old leaves	OL	146.25±1.12 b	52.78±1.96 b	0.40±0.06 b	0.21±0.03 b
Root	RO	187.21±1.68 af	40.98±1.72 e	nd	nd
Stems	ST	386.89±5.32 c	96.68±2.69 a	0.31±0.02 bd	0.17±0.02 b
Stem bark	SB	195.05±3.44 ef	122.17±3.35 d	0.20±0.03 d	0.16±0.02 b
Seed capsules	SC	37.47±2.44 d	27.69±1.25 c	nd	nd
Flowers	FL	205.65±3.67 d	17.06±1.64 f	0.09±0.01 de	0.00±0.00 d

*All values are mean ± standard deviation (n = 3). Means with different letters within a column are significantly different ($P < 0.05$). GAE, gallic acid equivalents; dE, dry extract; nd, not determined.

Table 2. DPPH radical scavenging activity (reaction time from 5 to 30 min), inhibition of linoleic acid oxidation and reducing power of methanolic extracts of *Hypericum foliosum* anatomical parts *.

Sample	Reference	FRSA (%)						Inhibition LA oxidation (%)	Reducing power EC_{50} (µg/mL) †
		5 min	10 min	15 min	20 min	25 min	30 min		
BHT		10.66±5.17	15.57±2.14	17.11±1.49	18.92±2.32	20.41±3.13	22.34±4.80 a	88.18±2.92 a	8.44±1.35 a
Aerial parts	AP	69.18±13.42	84.48±7.63	88.72±4.80	90.52±2.92	90.91±1.40	90.90±0.85 b	72.62±5.58 b	31.41±0.50 b
Young leaves	YL	72.54±5.98	76.93±4.57	79.45±4.3	80.56±3.06	81.95±2.98	83.19±3.27 c	42.38±4.00 c	44.93±0.71 c
Old leaves	OL	64.85±10.73	74.67±5.57	79.29±5.29	81.30±4.41	83.03±3.65	84.65±3.03 c	82.60±3.88 a	45.44±0.91 c
Root	RO	88.11±12.49	92.63±4.39	94.19±1.88	94.54±1.73	94.51±1.74	94.45±1.76 bd	78.81±3.25 ab	44.44±0.30 c
Stems	ST	66.92±12.69	83.46±10.21	89.64±6.48	93.06±4.38	94.42±3.26	95.57±2.42 d	89.17±2.09 a	6.59±1.88 a
Stem bark	SB	82.30±12.98	91.28±3.07	92.59±2.14	92.34±2.25	92.13±2.38	92.04±2.44 bd	86.23±0.82 a	25.65±0.78 b
Seed capsules	SC	43.90±4.83	48.90±6.09	51.51±6.52	54.32±6.78	55.43±6.45	58.86±6.79 e	29.16±6.79 c	91.39±1.76 d
Flowers	FL	59.90±4.28	83.37±3.67	90.67±2.71	92.12±2.39	92.40±2.21	92.69±2.21 bd	39.68±0.33 c	108.69±6.97 e

* All values are mean ± standard deviation (n=3). Means with different letters within a column are significantly different ($P<0.05$). † EC_{50}, effective concentration at which the absorbance is 0.5.

the extract to scavenge free radicals in solution. The results (Table 2) of the FRSA of different parts of HF revealed that AP, RO, ST, SB and FL extracts presented values above 90% being the ST extract the most active (95.57%), after 30 min of reaction time. Percentages above 90% can be considered as a full absorption inhibition of DPPH, because after completing the reaction the final solution always possesses some yellowish colour and therefore its absorption inhibition compared to colourless methanol solution cannot reach 100% (Miliauskas et al., 2004).

The YL and OL extracts have shown a high activity with no significant difference (83.19 and 84.65%, respectively). The SC extract presented the lowest ability to react with DPPH (58.86%). The BHT, used in the same conditions, as a positive control, presents a FRSA of 22.34% which is a very low value compared with the plant material. The knowledge of the kinetics of the DPPH consumption is also important because free radicals in the organism are short-lived species, what implies that the impact of a substance as an antioxidant depends on its fast

reactivity towards free radicals. Determinations of all samples were performed in triplicate using a single concentration (33.3 µg/ml), and during the time period of 5 to 30 min (Table 2). The extracts react very quickly with the DPPH in solution, being the RO and SB extracts the most active towards the first 5 min of reaction time. Furthermore, the time needed to reach the 50% DPPH consumption is under the first 5 min for all samples, with the exception of the SC extract, which reveals that these plant extracts are very effective antioxidants in these conditions. The results of FRSA for all plant materials and BHT show a slightly increased value with increasing reaction time, particularly from 10 to 30 min.

In order to evaluate the radical scavenging stability, the plant extracts were stored at -20ºC for two months and the DPPH assay was repeated 4 times, for each sample, over that period. The FRSA level was maintained constant for all samples, except for the SC extract, that show a decrease of *ca* 10% over that period.

These results are in accordance to those published in literature by different researchers, indicating that the rich profile of phenolic compounds is the main responsible for the free radical scavenging properties of *Hypericum* species (Silva et al., 2005; Silva et al., 2008). Furthermore, Gioti et al. (2009) was able to conclude that chlorogenic acid is the compound contributing the most to the DPPH antioxidant activity of *H. perforatum* on both organic and aqueous extracts. On the contrary, less significant is the contribution of flavonoids to the DPPH scavenging activity. According to previous preliminary chemical studies on the phenolic profile of HF methanolic extracts, the FRSA could be related to the high content of hydroxycinnamic acids.

β-Carotene/LA assay

The antioxidant activity of the extracts was tested with respect to the prevention of β-carotene/LA system oxidation. Lipids, such as LA, form peroxyl radicals in the presence of reactive oxygen species and O_2. The damages promoted by these peroxyl radicals affect the β-carotene colour and can be reduced in the presence of other antioxidants in solution (Dapkevicius et al., 1998; Moon and Shibamoto, 2009).

The potential of the plant extracts under study to inhibit lipid peroxidation was measured after two hours of incubation at 50ºC and compared with the antioxidant BHT under the same conditions.

The HF anatomical parts presented considerable differences in their activity that varied from 29.16 to 89.17% (Table 2). Since, our results can be divided into three distinct groups. First, the OL, RO, ST and SB extracts presents significant activities and are statistically compared to the reference compound BHT which presented a value of 88.18%. The AP extract presented a slightly lower activity (72.62%). Finally, the SC, FL and YL extracts presented the lowest activities (29.16, 39.68 and 42.38%, respectively).

Reducing power assay

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). Table 2 shows the reducing power of the extracts obtained as EC_{50} value (µg/ml) by interpolation from linear regression analysis of concentration vs. Abs at 700 nm. The reducing power was dose-dependent and the differences among the various methanolic extracts are evident. Since the ST extract presents a reducing power several times higher than the other plant extracts and statistically similar to the reference antioxidant BHT (EC_{50} value of 8.44 µg/ml). The AP and SB extracts are at least four times less active than the ST extract. The YL, OL and RO extracts showed similar activities (EC_{50} values of ~45 µg/ml). The SC and FL extracts were much less active than the other plant parts with EC_{50} values of 91.39 and 108.69 µg/ml, respectively.

Superoxide anion scavenging activity

In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of Abs at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The scavenging activity of the HF extracts, at concentrations from 31.3 to 125 µg/ml, was evaluated against the activity of gallic acid (Figure 1). The results are dose-dependent and vary from 5.15 to 60.58% with the majority of the samples showing activity under 30% at 125 µg/ml. At this concentration, the scavenging effect of HF anatomical parts decreases following the order: ST > SB > OL ~ YL > AP > RO > FL > SC. A weak scavenging effect was observed for all the extracts with the exception of ST extract (60.58%) which presents an EC_{50} (effective concentration at which 50% of radicals are scavenged) of 103.78 µg/ml. These results are not significant, since gallic acid, used as reference antioxidant in this test, prevented the formation of the chromophor at rates above 90%, even at the lowest concentration. Although the HF extracts had high FRSA (DPPH), its scavenging effect on superoxide anion was relatively weak.

Ferrous ion-chelating ability

It is known that Fe^{2+}generates lipid peroxidation through the Fenton reaction or by accelerating the dissociation of lipid hydroperoxides to the respective peroxy- and alkoxy-radicals (Gioti et al., 2009). Since, the amount of

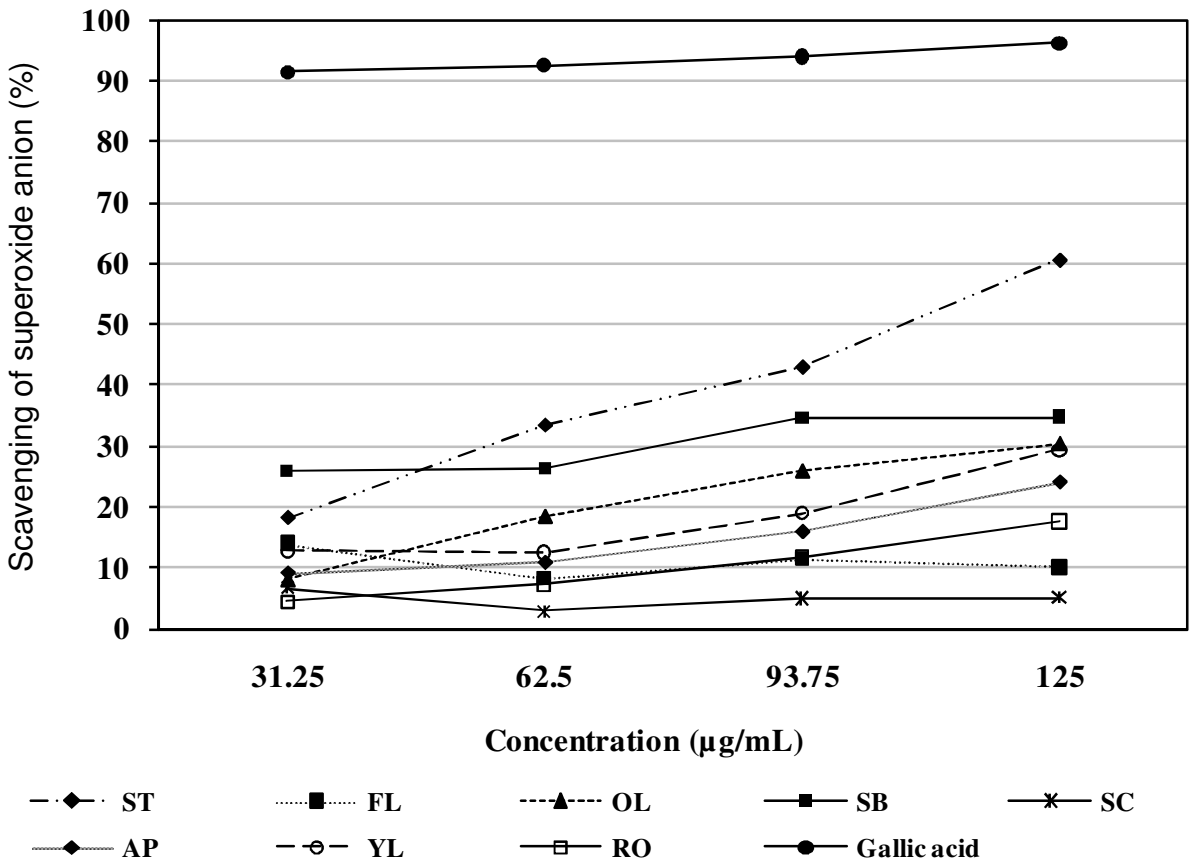

Figure 1. Superoxide anion scavenging activity of methanolic extracts of *Hypericum foliosum* anatomical parts in comparison with gallic acid.

chelating agents into the extracts is substantial for the reduction of free transition metals which give rise to catalytic lipid peroxidation. Thus, the ability of the extracts to bind with Fe^{2+} in the presence of ferrozine was compared with that of EDTA, which is a strong chelating agent. As shown in Figure 2, the % of iron chelating ability of HF extracts was dose-dependent (from 50 to 200 µg/ml) for the most active extracts. The percentage of metal scavenging capacity at 200 µg/mL for EDTA and the extracts decreased in the order of EDTA > SB > OL > AP > ST > RO > YL > SC ~ FL, varying from 99.24 (EDTA) or 45.3 (SB) to 11.62%. Although the chelating activity of the extracts was shown to be lower than EDTA's, the results obtain are not negligible for a natural product since different authors reports plants with chelating properties at much higher concentrations (Huang et al., 2006; Tsai et al., 2006).

BSA oxidative damage assay

Oxidative damage of proteins is attributed to the presence of free radicals leading to the formation of carbonyl compounds (Levine and Stadtman, 2001). The ability of the extracts to prevent protein oxidative damage was evaluated based on the inhibition of carbonyls formation promoted by the Fenton reaction. Here, BHT was used as reference compound at concentrations of 62.5 to 250 µg/ml. BHT showed an increase in the antioxidant activity with increasing concentration and reached a maximum value of 15.97% antioxidant protection (Figure 3). Six of the HF extracts under study presented an increase in the pro-oxidative activity with increasing concentration. Thus, at concentrations of 250 µg/ml, the pro-oxidative activities decreased in the order of ST > AP > RO > SB > YL > SC, varying from 64.50 to 19.55%. On the other hand, the OL and FL extracts presented a low positive effect which was maximum at 125 µg/ml (8.05 and 5.84%, respectively). These results are not surprising since other researchers have noticed that several other plant extracts, or pure antioxidants, scarcely prevent metal-catalysed oxidation of proteins (Gioti et al., 2009; Makris and Rossiter, 2001). Besides, phenolic substances are known to bind proteins or amino acids, thus exhibiting reduced activity (Arts et al., 2001). The pro-oxidant activity of the extracts is a result of the ability to reduce metals, such as Fe^{3+}, to forms that react with O_2 or H_2O_2 to form initiators of oxidation. As a

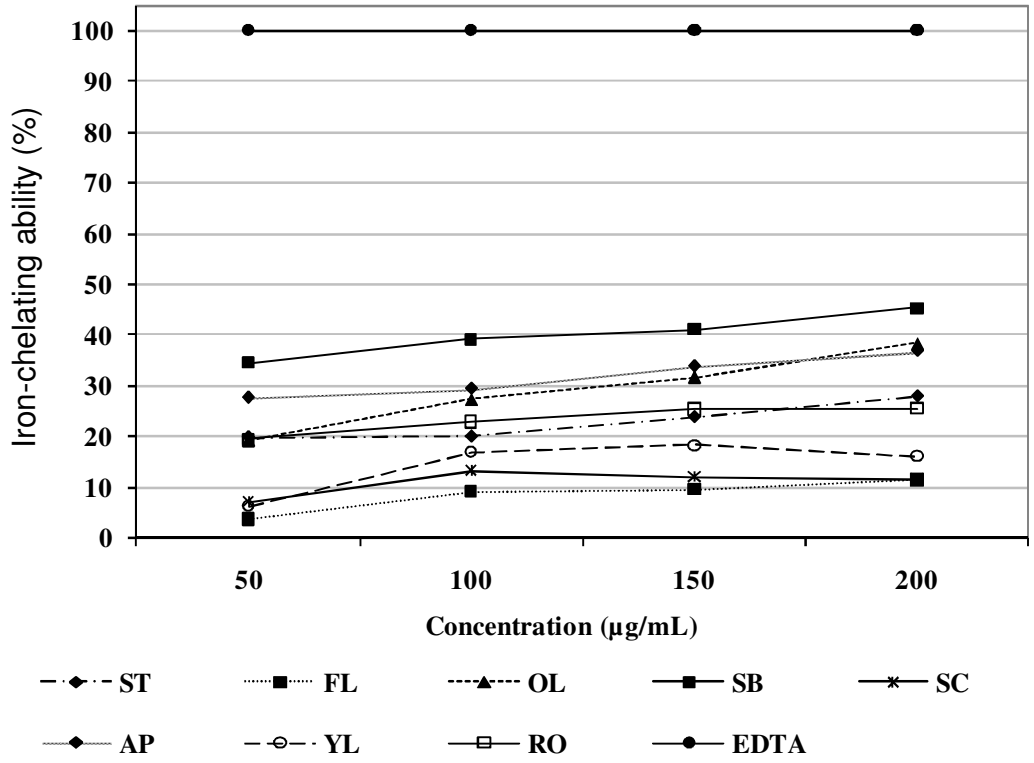

Figure 2. Ferrous ion-chelating ability of methanolic extracts of *Hypericum foliosum* anatomical parts in comparison with EDTA.

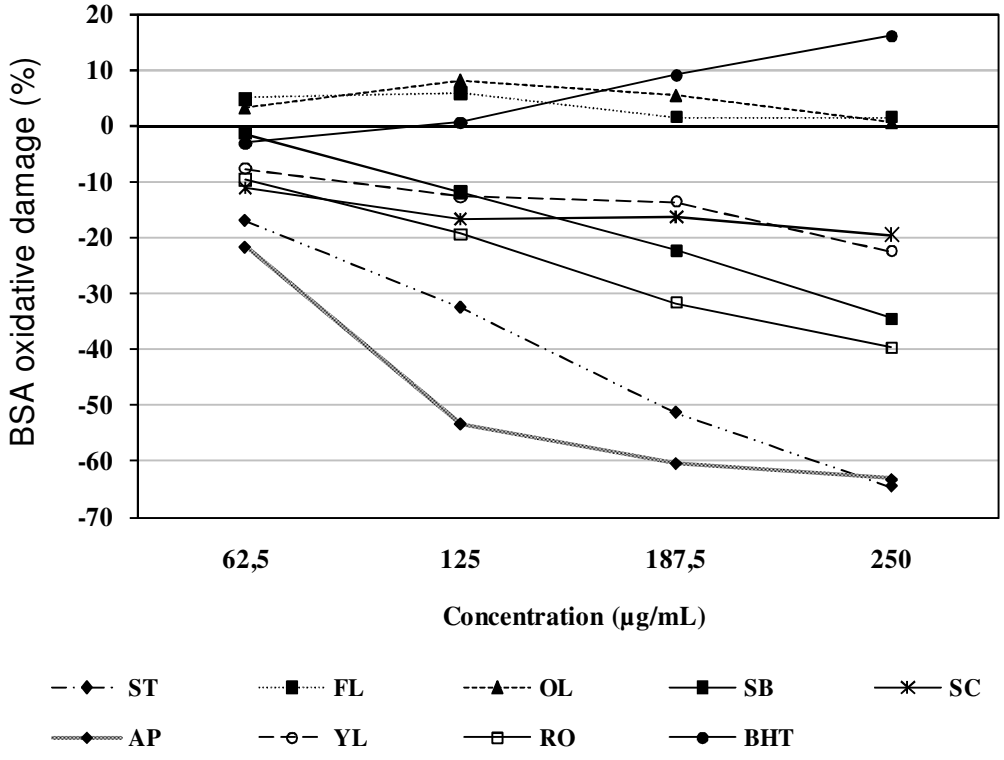

Figure 3. BSA oxidative damage of methanolic extracts of *Hypericum foliosum* anatomical parts in comparison with BHT.

general rule, the antioxidants extracted from plants can show pro-oxidant activity at low concentration and antioxidant activity over certain critical values, although the opposite effect is known, that is, antioxidant at low concentration and pro-oxidant at high concentration (Moure et al., 2001).

Correlation analysis of the measurements

It is widely accepted that the antioxidant activity of a plant extract is correlated to its phenolic content with several authors showing this correlations by different statistic approaches (Miliauskas et al., 2004; Velioglu et al., 1998; Wang et al., 2009), although some researchers question this possible correlation or simply are unable to find it (Bocco et al., 1998; Kähkönen et al., 1999). In order to elucidate the correlation between the phenolic and carotenoid content of HF samples and their antioxidant activity, linear regression analysis was performed, quoting the correlation coefficient of each of the antioxidant tests performed. Although the few number of samples (n = 8), it was obvious that the phenolic content in the extracts showed a satisfactory and significant correlation ($P < 0.05$) with FRSA ($r = 0.756$) and inhibition of LA oxidation ($r = 0.618$), and a strong significant ($P < 0.01$) correlation with the reducing power and superoxide anion activity results. The results prove the importance of phenolic compounds in the antioxidant behaviour of the extracts and also show that they contribute significantly to the total antioxidant capacity. No significant correlation could be found between phenolic content and the inhibition of the oxidative damage of protein ($r = -0.520$) or the chelating ability ($r = 0.296$), indicating that phenolic compounds in HF extracts do not appear to be effective iron chelators. Furthermore, total carotenoid content is positively correlated with the inhibition of LA oxidation ($r = 0.720$), reducing power ($r = 0.781$) and superoxide anion activity ($r = 0.697$). A strong and significant positive correlation between the total carotenoid content and the chelating ability was observed ($r = 0.815$; $P < 0.01$).

As these compounds are not important iron-chelating agents, by absence of the phenolic-type structure (Wang et al., 2010), the statistical value obtained should be analysed carefully. Since the measurement of carotenoid content is based on the Abs at 470 nm, it is possible that the iron-chelating ability is related to the concentration of a non carotenoid compound that presents a visible band near 470 nm. However, these correlations show that phenolics and carotenoids are undoubtedly responsible for the majority of the antioxidant properties of HF methanolic extracts, with the exception of chelating ability that could be related to other substances present in the extracts, such as polysaccharides, proteins or peptides. A similar conclusion was reported by Wang et al. (2009) who stated that the iron-chelating ability of water and70% acetone extracts of Icelandic seaweeds exhibited poor correlations with both TPC and other antioxidant activities, such as FRSA.

For a clearer arrangement the results of the different measurements were grouped in a manner that assigned similar behavior using the hierarchical agglomerative cluster analysis. Figure 4 shows both positive and negative correlations between the different antioxidant assays. For instance, the BSA oxidative damage was well separated from the results of the other assays. The reducing power and superoxide anion activities are strongly related ($r = 0.946$; $P < 0.01$) with each other and within a cluster with the total phenolic content. Positive correlations were also observed between the β-carotene/LA assay with the iron-chelating ($r = 0.850$) assay.

According to Figure 4, the total carotenoid content is grouped with these two assays. These correlations between the assays imply that some of the samples present higher overall results and other lower overall results. Since, the best overall results were obtained for the ST followed by SB and RO extracts. SC and FL extracts presented the lowest overall antioxidant scores.

Conclusions

The results reveal that when the antioxidant activity determination is carried out by a single method the antioxidant potential could be underestimated, since significant differences in phenolic, carotenoid content and antioxidant activities were observed.

It should also be noted that the reaction conditions of the different antioxidant assays may have a great influence on the effectiveness as antioxidant of the various plant extracts. From the eight plant parts analysed, it can be concluded that stems has the best overall antioxidant results, which may be accounted by the high phenolic content.

Based on these analyses, it can be suggested that the HF methanolic extracts are potent radical scavengers and primary chain-breaking antioxidants. However, no encouraging results were obtained regarding the inhibition of the oxidative damage of proteins, with six extracts promoting the damage of proteins exhibiting a functional pro-oxidant role. Since no pharmacological reports of the use of this *Hypericum* species are known, *in vivo* toxicity evaluation studies must be performed prior to any kind of commercial use of this plant. However, these results revealed a promising plant, especially its lower parts (stems, stem bark and root) that showed powerful antioxidant with different modes of action, for further evaluation of its potential applications.

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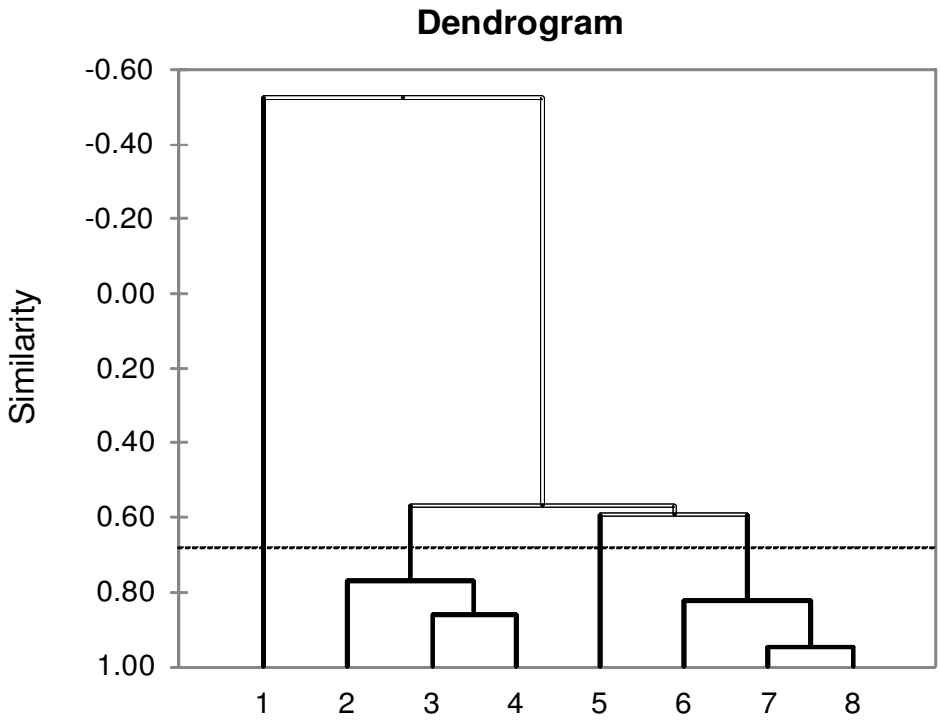

Figure 4. Dendrogram of the hierarchical agglomerative cluster analysis based on Pearson correlation similarities indicating the grouping obtained for the results of the antioxidant assays *versus* the total phenolics and total carotenoids. Legend: 1 - BSA oxidative damage; 2 - total carotenoids; 3 - inhibition LA oxidation; 4 - ferrous ion-chelating ability; 5 - FRSA; 6 - total phenolics; 7 - reducing power; 8 - superoxide anion activity.

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