ANTIOXIDANT ACTIVITY IN SOUTHERN BRAZIL *HYPERICUM* SPECIES

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ABSTRACT

The present study was conducted to assess the antioxidant activity of *Hypericum* species endemic to South Brazil, *H. caprifoliatum, H. carinatum, H. myrianthum* and *H. polyanthemum*. The free radical scavenging properties of plant extracts were evaluated employing different methodologies, including the bleaching of a stable free radical (2,2-diphenyl-1-picrylhydrazyl, DPPH) and the peroxyl reactivity indexes TRAP (Total Reactive Antioxidant Potential) and ORAC-pyrogallol red (Oxygen Radicals Absorbance Capacity). A fair correlation was found between total phenol content determined by Folin-Ciocalteau and DPPH consumption, both in crude methanol and n-hexane extracts. In particular, *H. myrianthum* and *H. caprifoliatum* showed the highest TRAP and ORAC-pyrogallol red values, respectively. This would imply that *H. myrianthum* contains a larger amount of antioxidants of lower reactivity.

Key Words: *Hypericum*; polyphenols, antioxidant activity; DPPH; TRAP; ORAC.

INTRODUCTION

Oxidative stress has been implicated in the pathogenesis of several neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases.[1,2] The lack of effective therapeutic agents for the treatment of these disorders has led to an intensive search of therapeutic alternatives based on the control of oxidative stress-induced neuronal damage. There is evidence that abnormalities in lipids may cause overproduction of reactive oxygen species (ROS) and/or decrease of the antioxidant activities. These phenomena may be related to the pathophysiology of major depression.[3] In the last years, several studies have been devoted to evaluate the effect of antioxidant supplementation on the prevention of oxidative stress conditions. In this context, the identification and development of new compounds, able to prevent and/or diminish ROS-induced damage, is of great interest.

Recent studies have shown that polyphenols and benzophenones, as well as plant extracts rich in these compounds, can exert antioxidant actions.[4-7] Polyphenols, and in particular flavonoids, are usually recognized as the compounds which are responsible for most of the plant extracts antioxidant activity.[8-12] The antidepressant activity of *Hypericum perforatum* (Guttiferae)[13] and evidence on the antioxidant activity of *Hypericum* species *in vitro,*[14-22] has encouraged us to further survey the chemical composition of several *Hypericum* species native to southern Brazil. Recently, some species were studied in our laboratory, affording a variety of phenolic compounds, such as benzopyrans,[23] benzophenones,[24] flavonoids[25] and phloroglucinol derivatives.[26,27] Some of the species were evaluated for antidepressant,[28] antiproliferative,[29] antimicrobial,[25,30] and analgesic activity.[31] Recently, it has been also shown a significant monoamine oxidases MAO-A and MAO-B inhibitory activity for 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP_3), a compound present in *Hypericum polyanthemum* non polar extracts.[32] Furthermore, the antioxidant capacity of several compounds present in *Hypericum* such as uliginosin B, cariphenone A , cariphenone B, and flavonoids have been evaluated. [24]

In the present work, we have studied the free radical scavenging properties of four Brazil native *Hypericum* species. The interaction of the crude methanol extracts with peroxyl radicals was estimated using both TRAP (Total Reactive Antioxidant Potential) and ORAC (Oxygen Radical Antioxidant Capacity) methodologies. The crude methanol extract and fractions (methanol, dichloromethane and n-hexane) were evaluated in terms of DPPH radical scavenging ability in ethanol. Also, the total phenol content in the samples was estimated by Folin´s method.

Methodology

Plant Material

Aerial parts of *Hypericum caprifoliatum* Cham. & Schltdl. were collected in Viamão; *H. myrianthum* Cham. & Schltdl. and *H. polyanthemum* Klotzsch ex Reichard were collected in Paraíso do Sul and Caçapava do Sul, respectively; *H. carinatum* Griseb. was collected in Glorinha. The vouchers specimens were deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN).

The plant material was dried at room temperature and powdered. The extracts were obtained by static maceration employing methanol at room temperature. The procedure involved three methanol extractions of 24 hrs, using new solvent each time in order to improve the extraction efficiency. Fractions of different polarity were obtained from the dried and powdered plant materials by consecutive extractions in a Soxhlet apparatus, using n-hexane, dichloromethane and methanol, successively. The Soxhlet extractions in each solvent were performed during 12h. After extraction, the solvent was evaporated to dryness under reduced pressure and the initial volume was reconstituted with ethanol or methanol.

Total phenolics

Total phenol content in the extracts was determined according to the Folin–Ciocalteau colorimetric method,[33] using quercetin as standard.[34] Briefly, appropriate dilutions of the samples were oxidized with 0.2 N Folin-Ciocalteau reagent (Merck Darmstadt, Germany - 2N, diluted ten-fold). After 5 min, sodium carbonate (75 g/L) was added. The mixtures were incubated for 30 min and the absorbance of the resulting blue color measured at 765 nm using an ultraviolet-visible Hewlett Packard 8453 spectrophotometer. Results were expressed as milligrams of quercetin equivalents per gram of dry plant $(QE)_{plant}$ or per gram of dry extract $(QE)_{extract}$.

Antioxidant activity

Determination of DPPH radical scavenging activity

Samples were evaluated in terms of radical scavenging ability by kinetic assays measuring spectrophotometrically the bleaching of the stable free radical DPPH.[35] Ethanol solutions of the crude methanol extracts and fractions (25 µL) were poured in 60 µM DPPH solution (final volume of 3 mL). DPPH and extracts solutions were daily prepared. The assays were carried out in an ultraviolet-visible Hewlett Packard 8453 (Palo Alto, CA, USA). The absorbance was measured at 517 nm (ε_{517nm} = 11500 $M^{-1}cm^{-1}$). Temperature was controlled at 25.0 ± 0.2 ◦C. Measurements started immediately after mixing the solutions and the absorbance decrease was evaluated up to 600 seconds.

Total Reactive Antioxidant Potential (TRAP) assay

TRAP, total reactive antioxidant potential, was measure by luminol-enhanced chemiluminescence, according to the method of Lissi et al.[36] This method is based on that antioxidant addition to a solution containing luminol and a free radical source (2,2'-azo-bis(2-amininopropane)dihydrochloride, AAPH) quenches the solution chemiluminescence for a period that is proportional to the amount of antioxidants in the sample.[36] Chemiluminescence was measured in the out-of-coincidence mode in a liquid scintillation counter (Wallac

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1409) as counts per minute (CPM). The sample background was obtained by adding 3 mL of 10 mM AAPH in phosphate buffer 50 mM, pH 7.4, to a glass scintillation vial. Afterwards, 10 µL of luminol (4 mM) was added to each vial and the measured chemiluminescence was considered as the initial value. Ten µL of Trolox (6-hydroxy-2,5,8 tetramethylchroman-2-carboxylic acid, 320 or 160 µM), quercetin (320, 160, 120 or 80 µM in ethanol) or *Hypericum* dried crude methanolic extracts dissolved in ethanol (1.0; 0.75; 0.5; 0.25 and 0.125 mg/mL) were added and the chemiluminescence was measured as a function of the elapsed time. The time at which the chemiluminescence intensity recovers to 20 % of the initial value (induction time, IT) was measured.

Oxygen Radicals Absorbance Capacity (ORAC) assay

Oxygen Radicals Absorbance Capacity (ORAC) values of the samples were determined employing the procedure proposed by Lopez-Alarcón et al.[37] This methodology measures the ability of the antioxidants present in the tested sample to inhibit the bleaching of pyrogallol red (PGR) absorbance elicited by AAPH.

Stock solutions of dried crude methanolic extracts (*Hypericum caprifoliatum* 2.5 mg/mL; *H. carinatum*, *H. myrianthum* and *H. polyanthemum* 5 mg/mL) and Trolox were prepared in methanol immediately before their use. Stock solutions of AAPH (0.6M) and PGR ($1x10^{-4}$M) were prepared daily in phosphate buffer 75 mM pH 7.4:methanol (60:40). A reaction mixture, with final volume 3 mL in phosphate buffer:methanol, containing AAPH (10 mM), PGR (5 µM) and the sample (25 µL) was incubated at 37°C in the thermostatized cuvette of a UV-visible spectrophotometer Hewlett Packard 8453 (Palo Alto, CA, USA). The consumption of PGR, associated to its incubation in the presence of AAPH, was evaluated from the progressive absorbance decrease measured at 540 nm. Values of (A/A_0) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (A/A_0) reached a value of 0.2. ORAC values, expressed as micromoles of Trolox equivalents (TE) per gram of dry extract, were calculated employing equation [1]:

$$ORAC = f\,[Trolox]\,(AUC - AUC^{o})/(AUC_{Trolox} - AUC^{o}) \qquad [1]$$

where AUC is the area under curve in presence of the extract, integrated between time zero and 80 % of probe consumption; AUC^{o} is the area under curve for the control; AUC_{Trolox} is the area under curve for Trolox, and f is a factor that takes into account the dilution of the extract. It is important to point out that ORAC values are independent of the additive concentration considered, because there is a linear relation between the AUC and the amount of the tested sample employed in the assay (data not shown). All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Determination of total phenols content

The amount of dry extract obtained from *Hypericum* species depend both on the employed solvent and the species considered. These data (**Table 1**) show that the amount of compounds in the crude extract are similar in the four species considered (between 27 to 36 grams of dry extract per 100 grams of dry plants), and that only a small fraction of the material is extracted with the non-polar solvents. This indicates that most of the material present in the crude extract corresponds to compounds of relatively high polarity.

Table 1: Total amount of extracts in *Hypericum* species expressed as grams of dry extract by 100 grams of dry plant.

Species	Crude extract	MeOH (*)	CH_2Cl_2 (*)	*n*-Hexane (*)
H. caprifoliatum	27.0 ± 1.4	21.7 ± 1.2 (80)	0.7 ± 0.1 (2.6)	2.7 ± 0.2 (9.9)
H. carinatum	33.6 ± 1.1	27.5 ± 1.9 (81.8)	2.8 ± 0.5 (8.3)	2.2 ± 0.3 (6.5)
H. myrianthum	27.7 ± 2.3	15.3 ± 1.4 (55.2)	1.2 ± 0.3 (4.3)	4.5 ± 0.3 (16.2)
H. polyanthemum	36.1 ± 1.1	32.2 ± 1.3 (89.2)	2.0 ± 0.1 (5.5)	3.4 ± 0.2 (9.4)

(*) Values between parentheses indicate the percentage of mass in the corresponding extract, relative to the amount present in the crude extract.

Plant phenols constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers.[38,39] The Folin-Ciocalteau reagent was employed to evaluate the total amount of phenolic groups in the extracts.[33] The total amount of phenolic compounds in the extracts was calculated with a linear equation based on a standard curve obtained employing quercetin:

$$y = 0.13x + 0.04; \quad r^2 = 0.9993 \qquad [2]$$

where y is the absorbance and x is quercetin concentration (µM). The results for the extracts from *Hypericum* species, expressed as quercetin microequivalents (QE) per gram of dry extract, are given in **Table 2**.

Plant polyphenols are widely distributed in the plant kingdom and they are frequently present in large amounts.[40] From the *Hypericum* species evaluated, the highest proportion was found in *H. carinatum* crude extract, where phenols (expressed as quercetin) amounts to ca. 23 % of the weight of the dry extract. This amount to ca. 7.7 g of phenols per 100 grams of dry plant.

A relevant conclusion of the data collected in **Table 2** is that the fraction of phenols present in the extracts obtained in non polar solvents is considerable smaller than in the crude extract. The low extraction efficiency of non-polar solvents is further emphasized if the small amount of extracts in these solvents is taken into account (**Table 1**).

A peculiar feature of the data given in **Table 2** is the fact that the fraction of phenols titrated in the crude extract is smaller than that detected in the methanolic fraction in *H. caprifoliatum* and *H. polyanthemum*. This could be due to the presence in the crude extract of non-phenolic compounds of low polarity (such as terpenoids) that are not present in the methanolic extraction. This proposal is compatible with the rather small fraction of phenolic compounds present in the non-polar solvents. Furthermore, it is interesting to note that if the results are expressed in amount of phenols per 100 grams of dry plants, always the larger amount is present in the crude extract.

The lowest percentage of phenolic compounds is present in the n-hexane extracts of *H. polyanthemum* and *H. carinatum*. This can be related to an important contribution of benzopyrans, such as HP3, whose structures are devoid of phenolic groups or present only one of these moieties).[23]

The data collected in **Table 2** can be compared with that reported for *Hypericum perforatum* extracts. Zheng and Wang[38] have determined the presence of 0.28 g of phenols (taking gallic acid as reference) per 100 grams of fresh plant. Furthermore, in the methanolic extract of this plant, Škerget et al.[7] have reported the presence of 0.19 grams of phenols (expressed as gallic acid equivalents) per grams of extract and these values are similar to those reported in the present work.

Folin's values can be considered as a measure of the total amount of phenolic groups since the response of the method is almost directly proportional to the number of phenolic groups present in the sample. However, it has been reported that different phenols can give different responses.[33] In particular, the presence of two vicinal hydroxyl groups can lead to an increase in the response of the method.[41] In order to evaluate parameters to the free radical removal capacity of the different extracts we have performed a series of assays aimed to evaluate the antioxidant capacity of the samples.

Antioxidant activity

DPPH radical bleaching.

All extracts, when added to DPPH ethanolic solutions bleached the visible absorbance of the sample, indicating capacity to scavenge free radicals. The extent of DPPH bleaching elicited per a given amount of sample (i.e. an extract) has been considered as a measure of the amount of free radicals scavengers present in the sample.[42] Typical results are summarized in **Table 3**.

Comparison of these data with the phenols contents present in the extracts (**Table 2**), allows to conclude that the amount of DPPH bleached by the extracts correlates with the amount of phenols. In particular, *H. carinatum* extract presents the largest amount of phenols and the highest capacity to bleach DPPH radicals. This is evidenced when the amount of DPPH bleaching is plotted against the amount of titrated phenols (**Figure 1**). A fair correlation is observed between both measurements that includes data obtained in n-hexane and crude extracts ($r = 0.93$, $p = 5 \times 10^{-4}$). Furthermore, the slope of the plot is near two, the expected theoretical value if it is considered that each quercetin molecule is able to bleach two DPPH radicals.

In *H. carinatum* and *H. polyanthemum* extracts,

$$(\text{Crude/n-hexane})_{DPPH} >> (\text{Crude/n-hexane})_{Folin}$$

Table 2: Total amount of phenol groups in *Hypericum* species expressed as quercetin microequivalents (QE) per gram of dry extracts or per 100 grams of dry plants.

Species	Crude extract		MeOH		CH_2Cl_2		n-Hexane	
	$(QE)_e$	$(QE)_p$	$(QE)_e$	$(QE)_p$	$(QE)_e$	$(QE)_p$	$(QE)_e$	$(QE)_p$
H. caprifoliatum	448 ± 7	12100 ± 200	556 ± 6	12000 ± 130	233 ± 12	158 ± 6	258 ± 2	685 ± 5
H. carinatum	756 ± 1	25300 ± 46	578 ± 2	15900± 60	138 ± 12	380 ± 35	124 ± 2	276 ± 5
H. myrianthum	478 ± 10	13200± 280	650 ± 3	9900 ± 40	243 ± 4	298 ± 4	287 ± 11	1280 ± 50
H. polyanthemum	458 ± 2	16500 ± 80	462 ± 2	14800 ± 60	297 ± 3	605 ± 6	143 ± 2	480 ± 7

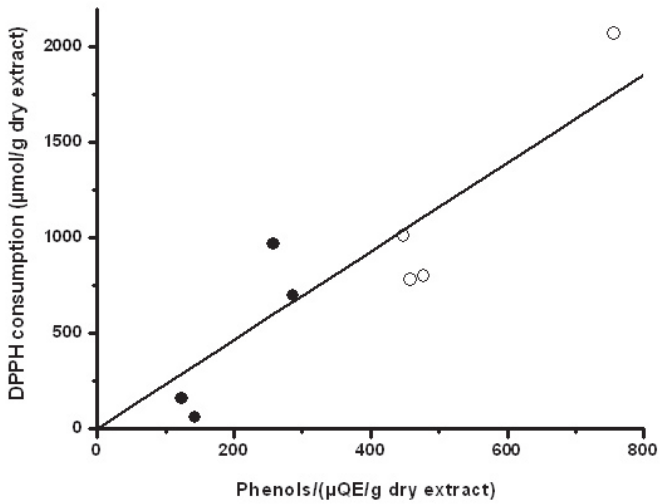

Figure 1:
DPPH consumption *vs* total phenol content. DPPH consumption was expressed as µmol of DPPH /g dry extract consumed after 600 s of reaction. Crude extract (○); hexane extract (●).

These results could indicate that, in n-hexane extracts, there are a large proportion of phenols of low reactivity. In order to test this possibility we measure the fraction of DPPH bleached in a short reaction time (50 s). This fraction is considerably smaller in the n-hexane extract (25 %) than in the crude extract (ca. 42 %) implying that, at least in *H. carinatum* and *H. polyanthemum* n-hexane extracts there is a large proportion of phenolic compounds of relatively low reactivity.

Table 3: Amount of DPPH radicals bleached (micromols) per gram of dry extract. The values given correspond to the average of three independent determinations and were measured after 600 sec of reaction.

Species	Crude extract	n-hexane extract
H. caprifoliatum	1020 ± 12	969 ± 33
H. carinatum	2070 ± 55	1656 ± 4
H. myrianthum	800 ± 10	707 ± 20
H. polyanthemum	784 ± 32	58 ± 1

Total Reactive Antioxidant Potential (TRAP) assay

Total Reactive Antioxidant Potential (TRAP) of the crude methanol extracts were evaluated by a procedure based on the quenching of luminol chemiluminescence.[36] Antioxidants can inhibit this chemiluminescence, giving an induction time which is directly proportional to the total antioxidant potential. The effect of the four crude methanol extracts, quercetin and Trolox is shown in **Figure 2**.

The chemiluminescence CL following the plant extracts or quercetin addition is qualitatively different to that obtained when Trolox is used. In particular, the rise of the CL intensity after the induction time is considerably faster in Trolox assays. This difference can be ascribed to the presence of groups of relatively low reactivity, both in quercetin and the plant extracts.[43,44]

The data given in **Fig. 2** allow an evaluation of the concentration of Trolox equivalents present in the extracts of the four species analyzed.

$$TRAP = (t_i / t_{Trolox})\ f\ [Trolox] \quad [3]$$

where t_i and t_{Trolox} are the induction times measured for the sample and Trolox, respectively, and f is a factor that takes into account the extract dilution in the measuring vial. The data obtained for this procedure are given in **Table 4**.

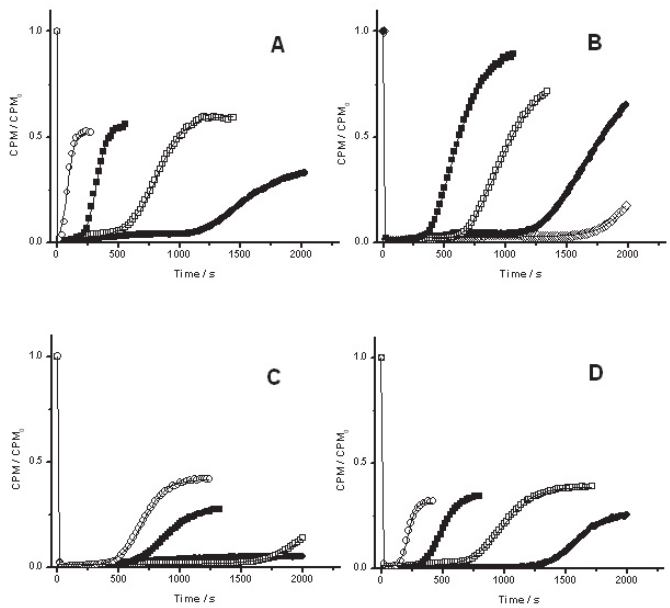

Figure 2:
Effect of *Hypericum* species extracts (methanolic) on the chemiluminescence (cpm) emitted from solutions comprising luminol (13 µM) and AAPH (10 mM). Graphic A: *Hypericum caprifoliatum*; graphic B: *Hypericum polyanthemum*; graphic C: *Hypericum myrianthum*; graphic D: *Hypericum carinatum*. Amounts added: 0.125 (◇); 0.25 (■); 0.5 (□); 0.75 (●); 1 (◇) mg/mL. Extracts were added after the initial intensity measurement (t = 0).

Comparison of these data with that given in **Table 2** for the methanolic extracts allows to conclude that:

i) TRAP values are of the same order of magnitude than total phenols detected by Folin's. This would indicate that most phenols present in the extract are able to trap peroxyl radicals.

ii) There is not correlation between TRAP and Folin's values (r = 0.44; p = 0.55). This indicates that differences in stoichiometric factors, included in TRAP values, are more important than the differences between the amounts of phenols present in the extracts.

Oxygen Radicals Absorbance Capacity (ORAC) assay

Methodologies aimed to obtain Oxygen Radicals Absorbance Capacities (ORAC) index are frequently employed to characterize the radical trapping capacity of pure compounds and their complex mixtures.[37] In the present investigation we used pyrogallol red (PGR) as target molecule in the evaluation of the scavenging capabilities of the crude methanolic extracts through an ORAC-like methodology (ORAC-PGR), by following the decrease of PGR absorbance at 540 nm. **Fig. 4** shows the protection afforded by *H. caprifoliatum* to the bleaching of PGR elicited by its incubation in presence of AAPH. The linearity of AUC *vs* antioxidant concentration, obtained both for Trolox and the tested extracts (data not shown), implies that ORAC values are independent of the additive concentration considered. Values of ORAC obtained with crude methanolic extracts from *Hypericum* species employing PGR under the present conditions are included in **Table 4**. The ORAC values vary from 240 to 820 µmol of Trolox equivalents (TE)/g. Among the four *Hypericum* species evaluated, the highest ORAC value was found in *H. caprifoliatum* crude extract. This can be, at least partially, associated to the exceeding amounts of phenols present in this extract (**Table 2**).

Table 4: TRAP and ORAC values, expressed as Trolox microequivalents per gram of crude methanolic extracts from *Hypericum* species.

Species	TRAP µmol/g	ORAC µmol/g
H. caprifoliatum	350 ± 100	820 ± 79
H. carinatum	450 ± 45	347 ± 25
H. myrianthum	1050 ± 150	261 ± 64
H. polyanthemum	870 ± 90	240 ± 33

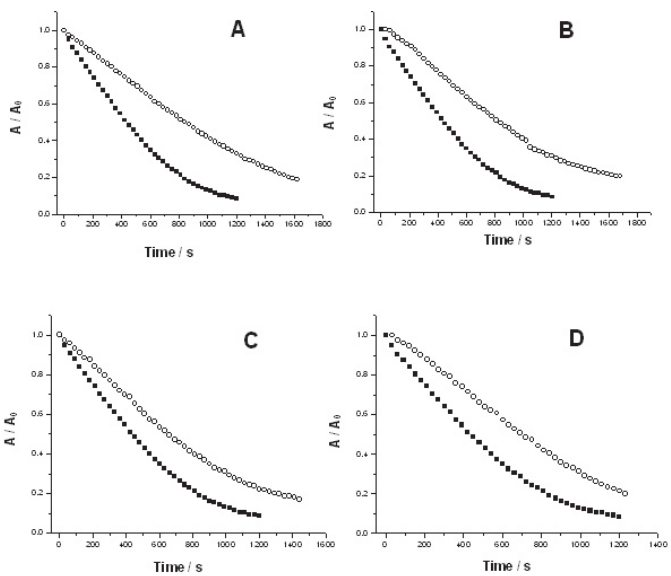

Figure 3:
Effect of *Hypericum* extracts on pyrogallol red bleaching mediated by AAPH. Pyrogallol red (5µM) was incubated in presence of both AAPH (10 mM), and *Hypericum* extracts at 37 ºC. Graphic A: *Hypericum caprifoliatum*; graphic B: *Hypericum carinatum*; graphic C: *Hypericum myrianthum*; graphic D: *Hypericum polyanthemum*. Control (■), 20.8 µg/mL (graphic A) or 41.7 µg/mL (graphic B, C,D) (◇).

The ORAC methodology is influenced both by the reactivity of the tested compound and the number of radicals that each molecule of the tested compound can remove. The relative importance of these factors depends upon the target molecule employed (phycoerythrin, fluorescein, c-phycocyanin and pyrogallol red), rendering ORAC values that are strongly dependent on the employed methodology. The efficiency of the tested compounds using PGR as a target molecule is considerably lower in the protection of PGR than in the protection of fluorescein as a target molecule. The competition for the peroxyl radicals under these conditions is more difficult and the tested compounds only reduce, in a concentration dependent way, the rate of the target molecule consumption. The use of PGR as a target molecule for peroxyl radicals provides ORAC indexes that are strongly influenced by the reactivity of the tested compounds.[37] Therefore, this approach it is a good choice for an estimation of the average reactivity of the antioxidants present in a complex mixture. In particular, a comparison of TRAP values (determined by the amount of antioxidants) and PGR-ORAC values (strongly conditioned by reactivity) can be employed to estimate the average reactivity of the antioxidants present in the tested sample. Employing this criterion, the data of **Table 4** would indicate that the extract bearing the more reactive antioxidants is *H. caprifoliatum*, while *H. myrianthum* and *H. polyanthemum* extracts comprise a large amount of relatively inefficient compounds.

The difference in the target molecule can explain the differences in ORAC values obtained in the present work with that reported in the literature. Zheng and Wang[38] have reported, for the aqueous crude extract of *H. perforatum* and ORAC index of 16.8 µM per gram of fresh plant employing R-phycoerithrin as target molecule. This value is considerably larger than those obtained in the present work for the crude methanolic extracts, ranging between 1.2 (*H. myrianthum*) and 6.8 (*H. carinatum*) µmols/g of dry extract.

In summary, this study have demonstrated that the amount of dry extract obtained from *H. caprifoliatum, H. carinatum, H. myrianthum* and *H. polyanthemum*, depend both on the employed solvent and the species considered. A fair correlation was found between total phenol content determined by Folin-Ciocalteau and DPPH consumption, both in crude methanol and n-hexane extracts. In particular, *H. myrianthum* and *H. caprifoliatum* showed the highest TRAP and ORAC-pyrogallol red values, respectively. This would imply that *H. myrianthum* contains a larger amount of antioxidants of lower reactivity.

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