EVALUATION OF THE ANTIOXIDANT ACTIVITY OF THE FLAVONOIDS ISOLATED FROM *HELIOTROPIUM SINUATUM* RESIN USING ORAC~FL~, DPPH AND ESR METHODOLOGIES

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ABSTRACT

The antioxidant capacity has been determined for a number of flavonoid compounds from *Heliotropium sinuatum*, a plant that grows in arid areas in the north of Chile. The methodologies used were: ORAC~FL~ (oxygen radical absorbance capacity - fluorescein), DPPH (2,2-diphenyl-2-picrylhydrazyl) bleaching and electron spin resonance (ESR). These compounds were studied in homogeneous and heterogeneous media. The results showed that the 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin are those with the highest antioxidant capacity.

Keywords: ORAC~FL~, ESR, DPPH, flavonoids, micelle, antioxidants.

INTRODUCTION

The increasing interest for natural products with beneficial effects on health, has led to a progressive increase in the study of secondary metabolites synthesized by plants and their biological activities. From this point of view, one of the most studied compounds are polyphenols, known for their properties as food antioxidants, agents preventive against cellular aging and diseases such as cancer.[1] To this respect, the specie *Heliotropium sinuatum* (Miers) is an endemic resinous bush that grows in the north of Chile in arid areas with extreme environmental conditions and produce resinous exudates from glandular trichome that covers the leaves and stems as a protection mechanism against the different types of stress.[2] Previous studies have shown that this plant is characterized because its resinous exudate consists mainly of flavonoids which would give to the resin mainly protection against oxidizing agents.[2]

Recently, it has been suggested that the flavonoids could protects lipids from oxidative damage that could affect the structure and/or function of biological membranes.[1] It is known that changes in the rheology of membranes, can affect biological events at the level of membrane cell, such as transport of metabolites and interaction membrane-receptor, among others.[3-4] The interaction between flavonoids and a bilayer is the result of distribution in lipid-aqueous interface or in the membrane hydrophobic inside. The more hydrophilic flavonoids interact by hydrogen bonding with the polar head groups at the lipid-water interface of membranes. This type of interaction may provide a level of protection for the bilayer from external and internal aggressors (*i.e.* oxidants) contributing to preserve the structure and function of biological membranes.[5] Therefore, the antioxidant activity may vary dramatically in the membranes and lipoproteins.

This has aroused great interest in the methods of quantification and identification of antioxidant capacity. There are different methods to evaluate the antioxidant capacity of flavonoids generally in homogeneous systems, such as methods based on the consumption of different radical in alcoholic solvents when flavonoids are added.[6] However, it is necessary to seek of new methods that incorporate heterogeneous environmental, for example the addition of micelles. These can mimic the inside non-polar and polar interfacial region relatively similar to the lipid bilayer of the cell membrane.

Based on these antecedents, we now report the results obtained on the evaluation of the antioxidant activity of the flavonoids isolated from *Heliotropium sinuatum* resin, using two different reaction media. Specifically, the used of an ethanolic solution of DPPH (homogeneous system) and a micellar solution of Triton X-100 (micro heterogeneous system). The antioxidant capacity of flavonoids was evaluated through the technique of ESR, ORAC~FL~ and bleaching of DPPH radical. Also was measured the distribution constants of these compounds in buffer/micelle media. The different affinities for the micelle, are reflected in the different antioxidant activities when changing the reaction medium.

Materials and methods

Plant material

Heliotropium sinuatum (Miers) samples were collected in III region, Chile, 29° 57' S, 71° W. A voucher specimen was deposited in the Herbarium of Natural History Museum of Santiago of Chile (ST2563).

Extraction and isolation of the flavonoids

The resinous exudate was extracted by immersion of the fresh plant material in dichloromethane for 60 s at room temperature and was concentrated to a sticky residue. The extract was purified by column chromatography (silica gel) using a hexano-ethylacetate step gradient yielding 3-*o*-methylgalangin, 7-*o*-methyleriodictyol, 3-*o*-methylisorhamnetin and Pinocembrine.[2]

Chemical Reagents

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2-diphenyl-2-picrylhydrazyl (DPPH), Fluorescein sodium salt (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich. Ethanol, Hydrogen Peroxide (H₂O₂) 30%, Octanol, Triton X-100 micelle, Sodium Hydroxide (NaOH), Sodium phosphate dibasic, sodium phosphate monobasic and *N,N*-dimethylformamide (DMF) were purchased from Merck.

Kinetic UV–Vis assay for DPPH.

A solution containing 75 μM of DPPH in ethanol and the different flavonoids were prepared in PBS buffer. UV absorbance of the solutions were measured at 517 nm.[7-8] The concentration in μM at which the absorbance at 517 nm decreases to 50% of its initial value was used as the IC~50~ value for each test solution.[7, 9] Kinetic studies were conducted by measuring the disappearance of DPPH in buffer under pseudo-first-order conditions at 25 °C. Determinations were conducted in triplicate. The same conditions were used for heterogeneous medium in presence of Triton X-100 (50 mM).

Oxygen radical antioxidant capacity-fluorescein (ORAC~FL~)

The ORAC~FL~ analyses were carried out on a Synergy HT multi detection microplate reader, from BioTek Instruments, Inc. (Winooski, USA), using white polystyrene 96-well plates, purchased from Nunc (Denmark).[10] Fluorescence was read from the top, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and 200 μL final volume. FL (70 nM, final concentration) and flavonoids solutions in buffer with a range of concentration between 10⁻⁷ to 10⁻⁹ M were placed in each well of 96-well plate. The mixture was pre-incubated for 15 min at 37 °C, before rapidly adding the AAPH solution (15 mM, final concentration). The microplate was immediately placed in the reader and automatically shaken prior to each reading. The fluorescence was recorded every 1 min for 120 min. A blank with FL and AAPH using buffer instead of the antioxidant solution were used in each assay. Five calibration solu-

tions using Trolox (10^{-4} to 10^{-6} M) as antioxidant were also done. The inhibition capacity was expressed as $ORAC_{FL}$ values and is quantified by integration of the area under the curve (AUC_{NET}). All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample.[11] The same procedure was performed for the heterogeneous medium.

Kinetic ESR Assays for DPPH

DPPH radical scavenging capacity of the flavonoids was determined by an ESR spectrometry method.[7-8, 12] We chose those flavonoids showing the higher and lower $ORAC_{FL}$ values, respectively. We followed the decrease in the intensity of the signal showed by DPPH radical. DPPH solution was in the order of µM and the flavonoids concentration was 100 times greater (mM) to ensure a pseudo-first-order decay. The same conditions were used for heterogeneous medium.

Hydroxyl radical scavenging assay using electron spin resonance (ESR)

Reactivity of all the flavonoids against the hydroxyl radical was investigated using the non-catalytic Fenton type method.[13-14] ESR spectra were recorded in the X band (9.5 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50-kHz field modulation, equipped with a high-sensitivity resonator at room temperature. Spectrometer conditions were: microwave frequency 9.80 GHz, modulation amplitude 0.90 G, time constant 81.92 ms and conversion time 40.96 ms. The scavenging activity of each derivative was estimated by comparing the ˙DMPO-OH adduct signals in the antioxidant-radical reaction mixture and the control reaction at the same reaction time, and is expressed as scavenging percent of hydroxyl radical.[11] To prepare the samples, 150 µL of DMF and 50 µL of NaOH (25 mM) were mixed, followed by the addition of 50 µL of DMPO spin trap (0.2 M stock concentration) and finally 50 µL of H_2O_2 30%. The mixture was put in an ESR cell and the spectrum was recorded after five minutes of reaction. All flavonoids were studied at a concentration of 8 mM.

RESULTS AND DISCUSSION

Kinetic UV–Vis assay for DPPH.

The IC_{50} value (concentration of compound at which 50% is consumed DPPH) for the antioxidant capacity of the different compounds was determined. IC_{50} values are summarized in Table 1.

Table 1. IC_{50} values in homogeneous medium for flavonoids studied.

Flavonoids	IC_{50} (M)
Naringenin	1.85×10^{-3}
3-*o*-methylgalangin	1.59×10^{-3}
7-*o*-methyleriodictiol	4.03×10^{-5}
3-*o*-methylisorhamnetin	8.77×10^{-5}
Pinocembrin	2.76×10^{-3}

From the values shown in Table 1, it is possible to establish the following trend in relation to the antioxidant capacity of flavonoids studied: 7-*o*-methyleriodictiol > 3-*o*-methylisorhamnetin > 3-*o*-methylgalangin > naringenin > pinocembrin. This trend is based on the number and position of phenolic hydroxyl groups present in the flavonoids. In general terms, the antioxidant activity of these compounds is mainly influenced by the number and positions of the hydroxyl groups in ring A and B, in addition to the conjugation present in rings B and C. Table 2 summarizes the IC_{50} values when performing the test in heterogeneous media.

Table 2. IC_{50} values in heterogeneous medium for flavonoids studied using Triton X-100 micelle.

Flavonoids	IC_{50} (M)
Naringenin	2.34×10^{-3}
3-*o*-methylgalangin	1.89×10^{-3}
7-*o*-methyleriodictiol	–
3-*o*-methylisorhamnetin	–
Pinocembrin	3.05×10^{-3}

It was not possible to determine the IC_{50} for compounds 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin because increasing their concentration does not

decrease the absorbance of DPPH. This behavior may be due to problems of distribution of the reactants in the micelle.

The naringenin, 3-*o*-methylgalangin and pinocembrin compounds follow the same trend observed in the homogeneous medium (Table 1). Therefore, the following relationship is obtained by using heterogeneous medium: 3-*o*-methylgalangin > naringenin > pinocembrin. Figure 1 shows the typical kinetic profile obtained for the decay of DPPH radical using naringenin as antioxidant.
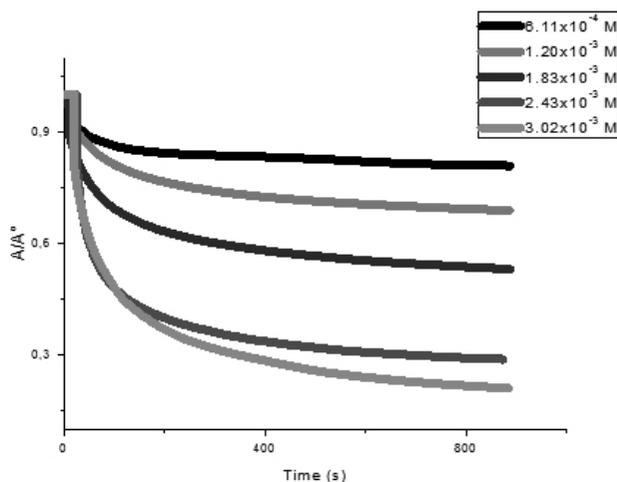

Figure 1. Shows the kinetic profile for naringenin as antioxidant in homogeneous media.

Oxygen radical antioxidant capacity-fluorescein ($ORAC_{FL}$)

Once IC_{50} values were obtained, we proceeded to perform the $ORAC_{FL}$ assay. Table 3 summarizes the values in Trolox equivalents for each of the studied compounds.

Table 3. $ORAC_{FL}$ values for the flavonoids studied in homogenous media.

Flavonoids	$ORAC_{FL}$ Value
Trolox	1.0 ± 0.01
Naringenin	4.19 ± 0.22
3-*o*-methylgalangin	7.18 ± 0.17
7-*o*-methyleriodictiol	14.62 ± 0.39
3-*o*-methylisorhamnetin	10.91 ± 0.18
Pinocembrin	3.22 ± 0.16

The values show that compounds 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin exhibit higher $ORAC_{FL}$ values that many widely used antioxidants (quercetin has an $ORAC_{FL}$ value of 7.28 ± 0.22 and is considered as one of natural flavonoids with higher antioxidant activity).

Thus, these results indicate that 7-*o*-methyleriodictiol is the compound with the highest antioxidant capacity, followed by 3-*o*-methylisorhamnetin, 3-*o*-methylgalangin, naringenin and pinocembrin being the compound with the lowest antioxidant capacity. We proceeded to perform this test in heterogeneous media. The values obtained in this environment are summarized in Table 4.

Table 4. Summarizes the obtained $ORAC_{FL}$ value in heterogeneous media for the antioxidant studied.

Flavonoids	$ORAC_{FL}$ Value
Trolox	1.0 ± 0.01
Naringenin	12.20 ± 0.16
3-*o*-methylgalangin	19.53 ± 0.25
7-*o*-methyleriodictiol	62.54 ± 0.32
3-*o*-methylisorhamnetin	48.97 ± 0.26
Pinocembrin	6.43 ± 0.19

Micellar ORAC$_{FL}$ values have the following high to low antioxidant capacity trend: 7-*o*-methyleriodictiol > 3-*o*-methylisorhamnetin > 3-*o*-methylgalangin > naringenin > pinocembrin. In order to explain these results in heterogeneous media (specifically we used triton X-100 micelle) we calculated free fluorescein concentration [FL].[15]

We obtained a value of 74% of free fluorescein. This indicates that the fluorescein is not a good alternative as a probe to evaluate the antioxidant capacity in heterogeneous media using the Triton X-100 micelle, and only 26% of fluorescein would be entering to the micelle. Probably there is not a complete interaction of the probe molecule with the antioxidant, which may explain the extensive delays that exists in the decrease in fluorescence intensity over time. Figure 2 shows the behavior previously described for naringenin and pinocembrin compounds in different medium.
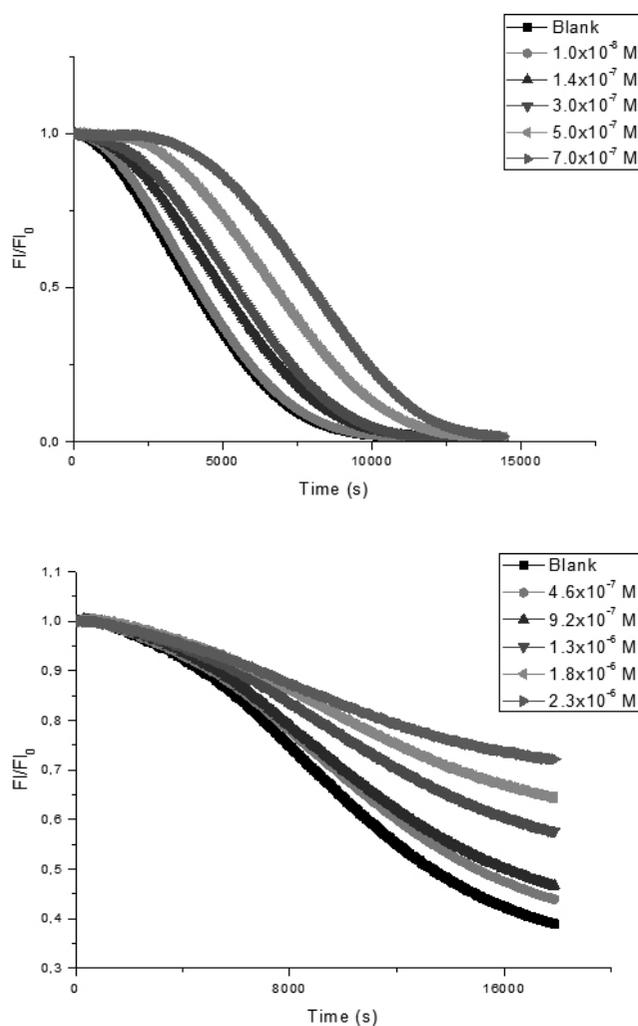
Table 5. Summarizes the values obtained for the selected antioxidants studied in ESR in homogeneous media.

Flavonoids	k_2 (M^{-1}s^{-1})
Naringenin	0.11 $\pm$ 0.01
7-*o*-methyleriodictiol	1.75 $\pm$ 0.14

Table 6 shows the decay values constants for the above mentioned compounds using heterogeneous medium. It is possible to realize that the trend obtained in a homogeneous medium remains when using the heterogeneous medium.

Table 6. Summarizes the values obtained for the selected antioxidants studied in ESR in heterogeneous media.

Flavonoids	k_2 (M^{-1}s^{-1})
Naringenin	0.17 $\pm$ 0.02
7-*o*-methyleriodictiol	10.54 $\pm$ 0.26

Figure 3 shows the kinetic profile in both medium for 7-*o*-methyleriodictiol. These profiles were used to obtain the values of the constants summarized in Table 5 and 6 respectively.
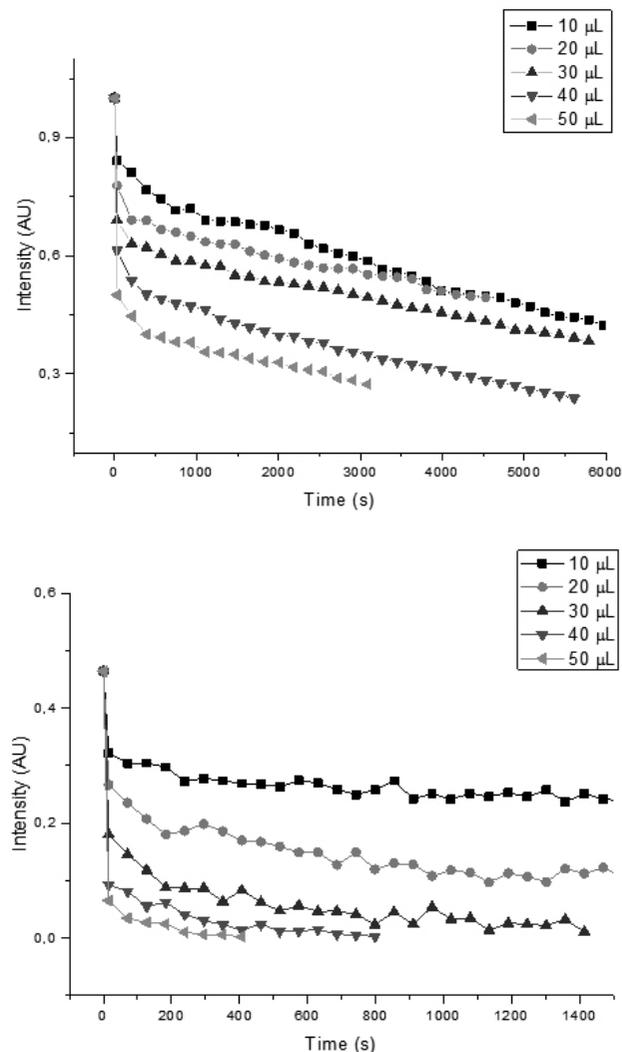

Figure 2. Left side shows the kinetic profile for naringenin in homogeneous media. Right side show the kinetic profile with extensive delays for pinocembrin in micellar media.

Kinetic ESR Assays for DPPH

Decay kinetics assays were performed for compounds: Naringenin and 7-*o*-methyleriodictiol in the presence of the DPPH radical. The following experimental conditions were used: DPPH (0.052 mM) and compound concentrations of 5.5 mM and 10.50 mM in the case of naringenin.

Table 5 shows the the decay values constants obtained. It is clearly, that the decay constant is much greater for 7-*o*-methyleriodictiol compared to naringenin. Indicating that 7-*o*-methyleriodictiol has a higher reactivity with DPPH radical.

Figure 3. Kinetic profile for 7-*o*-methyleriodictiol using ESR. Left side corresponds to a homogeneous media and right side shows the profile using heterogeneous media.

Hydroxyl radical scavenging assay using electron spin resonance (ESR)

The capacity of the studied compounds to quench the hydroxyl radical reactivity using a non-catalytic method was also determined. Table 7 summarizes the values expressed as percentage of decrease with respect to a blank solution.

Table 7. Summarizes the signal decrease expressed as percentage.

Flavonoids	% Decrease OH
Naringenin	47%
3-*o*-methylgalangin	59%
7-*o*-methyleriodictiol	70%
3-*o*-methylisorhamnetin	68%
Pinocembrin	24%

It is observed that the compound 7-*o*-methyleriodictiol quenched in a 70% the hydroxyl radical signal, proving to be the one with a higher reactivity towards this radical, being followed by 3-*o*-methylisorhamnetin with a very close value to 68%. Is possible to establish a trend in decreasing order of reactivity of quenching: 7-*o*-methyleriodictiol > 3-*o*-methylisorhamnetin > 3-*o*-methylgalangin > naringenin > pinocembrin. Figure 4 shows the spectra obtained when naringenin was used as antioxidant.
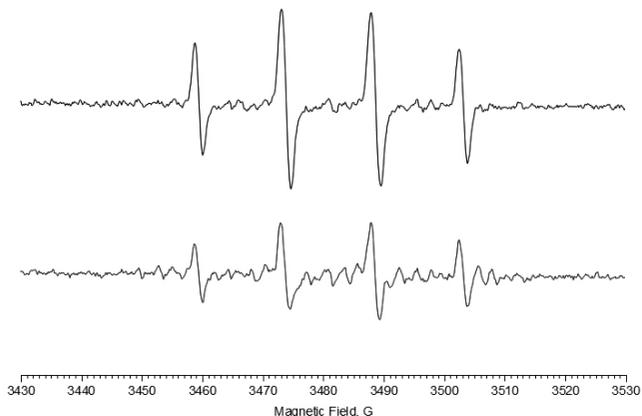

Figure 4. Upper shows the spectra for the blank solution. Lower shows the decrease of the signal when naringenin was added as antioxidant.

Based on the obtained results in homogeneous media, we propose the following decreasing order of antioxidant capacity for the compounds under study: 7-*o*-methyleriodictiol > 3-*o*-methylisorhamnetin > 3-*o*-methylgalangin > naringenin > pinocembrin. We explain these results mainly based on the number of phenolic hydroxyl groups which help to increase the antioxidant capacity. It is then noted that the studied compounds possess three hydroxyl groups (naringenin, 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin) and two hydroxyl groups (3-*o*-methylgalangin and pinocembrin). However, the number of hydroxyl groups is not sufficient to explain the improved antioxidant capacity, thus the presence of an *o*-dihydroxy structure in the B ring, confers more stability to radical form enhancing the delocalization of electrons. A double bond in conjunction with the 4-oxo function of the C ring. Hydroxyl substituents at position 3 and 5 with 4-oxo function on the rings A and C necessary to increase the antioxidant capacity. The presence of the double bond between carbons 2 and 3 of the C ring, because it enhances the resonance stabilization of the radical formed.

Thus, it is observed that pinocembrin is one of those that has less hydroxyl groups, these being located on C5 and C7 position of ring A. It has no double bond between C2-C3 carbons of the ring C, which would explain a lower capacity as an antioxidant. Naringenin bears a greater number of hydroxyl groups located at C5 and C7 of ring A and in position 4' of the ring B. This lower attributed to the radical resonance stabilization formed. Instead, 3-*o*-methylgalangin has less hydroxyl groups but has a double bond between positions C2 and C3 of the C ring, in addition hydroxyl groups at C7 and C3 position. The latter could be stabilized with the methoxy group at the C3 position enhancing antioxidant capacity, which would explain a better antioxidant capacity compared to naringenin.

Compound 3-*o*-methylisorhamnetin exhibits improved antioxidant capacity with respect to the aforementioned compounds because it has a double bond between carbons 2 and 3, it has a methoxy group at position C3 and finally a hydroxyl group at the C5 position. This latter group presents a lower influence with respect to the catechol group located in the B ring of compound 7-*o*-methyleriodictiol. This substituent improves antioxidant properties due to a higher stabilization of the radical caused by the abstraction of H atom at position C4', forming a hydrogen bond with the hydroxyl group at C3' position.

In order to rationalize the results between the different media used, we analyzed the effects of the medium used in the different measurements. It is possible to observe that the IC_{50} values obtained for the flavonoid do not allow for the differentiation of the antioxidant activity between the media used. Therefore, to compare the activity in the different media we used the variation of the initial absorbance (defined as $\Delta A_0 = A_0 - A_{10}$)

This parameter allows for the estimation of the initial velocity of DPPH radical consumption. The analysis of this parameter gives some idea of the efficiency of the compounds as antioxidants in different media. We determined the initial absorbance variation (during the first 10 seconds) depending on the concentration of the compounds. Thus, it is possible to observe a difference of ΔA_0 parameter throughout the concentration range studied in the different media used, demonstrating the influence of environment on the process speed. Figure 5 shows the initial absorbance variation for naringenin in both medium.
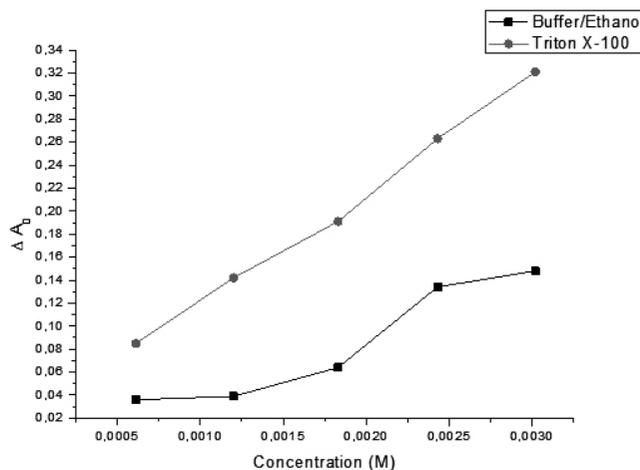

Figure 5. Initial absorbance variation (ΔA_0) for naringenin in both medium.

Then, these compounds are favored by the micelle, improving the process speed of reaction between these compounds and the DPPH radical. Most favored compounds using heterogeneous medium are: Naringenin, 3-*o*-methylgalangin and pinocembrin. For compounds 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin it was not possible to determine their IC_{50} values. From the results obtained through the $ORAC_{FL}$ method, we can argue that the tendency of antioxidant capacity found is maintained independently of the medium used. Finally, ESR measurements show an increase in speed when switching from homogeneous to heterogeneous medium environment.

CONCLUSIONS

Studied flavonoids from *H. sinuatum* showed an antioxidant activity which can be measured with different methods such as: DPPH, $ORAC_{FL}$ and ESR.

DPPH assay in terms of IC_{50} indicated that the flavonoid 7-*o*-methyleriodictiol has the highest antioxidant capacity against this radical. In contrast, using the DPPH assay in heterogeneous medium demonstrated that the IC_{50} method is not an accurate parameter to evaluate the antioxidant capacity of these flavonoids. In the $ORAC_{FL}$ assay, it is important that the fluorescent probe does not have a high affinity for the micelle, which can affect the results. ESR assay demonstrates that there is an increase in the decay rate and therefore in the reactivity of the flavonoids in a micellar medium. Reactivity towards the hydroxyl radical of the flavonoids studied presents the same trend obtained with the different assays used to determine the antioxidant capacity.

Based on these results it can be concluded that flavonoids here studied, are in general good antioxidant compounds: being 7-*o*-methyleriodictiol and 3-*o*-methylisorhamnetin those with the highest antioxidant capacity.

In addition, it is observed that these compounds conserve their antioxidant properties independent of the method and medium used.

ACKNOWLEDGEMENTS

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