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Antioxidant activity of gadusol and occurrence in fish roes from Argentine Sea

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ABSTRACT

The occurrence of the natural antioxidant gadusol (3,5,6-trihydroxy-5-hydroxymethyl-2-methoxycyclohex-2-en-1-one) was quantified in fish roes of three species from Argentine Sea: argentine hake (*Merluccius hubbsi*), brazilian sandperch (*Pinguipes brasilianus*) and argentinian sandperch (*Pseudopercis semifasciata*). Significant yields of the compound were found in the sandperchs. The antioxidant activity of the isolated metabolite in aqueous solution was assessed by ORAC and ABTS assays and compared with that of other known natural antioxidants. The results indicate that gadusol is a good breaker of chain reactions carried by peroxyl radicals. Besides, its ability to reduce radicals is comparable to that of ascorbic acid. On this basis, fish roes from brazilian and argentinian sandpearchs are proposed as useful sources of antioxidants for human consumption.

1. Introduction

Antioxidants are essential in preserving good health by their ability to delay or prevent the oxidation of biologically relevant molecules in living tissues (Halliwell & Gutteridge, 1999, chap. 3). In the last decades, the increasing interest in finding sources of natural antioxidants for food and cosmetics industries has lead to exploring diverse terrestrial and aquatic organisms (Cardozo et al., 2007; Peschel et al., 2006).

Recently, extracts from unused by-products such as head and viscera of salmon have been investigated as cheap sources of natural antioxidants (Wu & Bechtel, 2008). Besides, the accumulation of antioxidant molecules in fish gonads has also been studied mainly exploring the protection of embryos during the first stages of development (Martínez-Alvarez, Morales, & Sanz, 2005). In addition, fish roe products are widely consumed and are known for their nutritional value, although little is known about their chemical composition (Bledsoe, Bledsoe, & Rasco, 2003; Méndez, Fernández, Pazo, & Grompone, 1992). Cowey, Bell, Knox, Fraser, and Youngson (1985) have determined concentrations of ascorbic acid and other diverse antioxidants in the lipid phase of fish eggs, at clearly defined embryonic stages. Carotenoids in eggs from salmon and trout have been also assessed, in relation with their antioxidant properties (Czeczuga, Bartel, & Czeczuga-Semeniuk, 2002). However, the number of reports on water-soluble antioxidants in fish tissues is limited and the information on the individual components of the extracts is scarce. The antioxidant activities of aqueous extracts from eggs of salmon, trout, shark and herring have been analysed through the oxidative stability of polyunsaturated fatty acids in the liposome (Miyashita, Inukai, Ota, Sasaki, & Ota, 1999). The extracts from trout eggs have been separated according to molecular weight. Although the composition has not been identified, they have found that the lower molecular weight (<5000) fraction shows a higher activity.

A particular class of small-molecule antioxidants detected in many marine organisms belong to the group of the mycosporine-like amino acids (MAAs). This family of water-soluble molecules have been extensively studied in order to assess their physiological role. Especially, the photoprotective function of MAAs has attracted the interest of many researchers in connection to the increase of UV-radiation levels in the environment due to the stratospheric ozone hole (Conde, Churio, & Previtali, 2004; Shick & Dunlap, 2002). Among MAAs, those with a cyclohexenone structure (oxo-MAAs) such as the mycosporine-glycine, have proved to be

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significantly reactive towards radical-scavenging and singlet-oxygen quenching (Dunlap & Yamamoto, 1995; Suh, Lee, & Jung, 2003).

Gadusol (3,5,6-trihydroxy-5-hydroxymethyl-2-methoxycyclohex-2-en-1-one) and deoxygadusol (3,5-dihydroxy-5-hydroxymethyl-2-methoxycyclohex-2-en-1-one) are structural- and biosynthetically related to the MAAs (Fig. 1). They have been discovered in roes of some marine teleost fish (Chioccara, Della Gala, De Rosa, Novellino, & Prota, 1980; Grant, Plack, & Thomson, 1980; Plack et al., 1981). Eggs, ovaries and larvae of various marine invertebrates have also been found to contain either gadusol or deoxygadusol, or both (Bandaranayake, Bourne, & Sim, 1997; Bandaranayake & Des Rocher, 1999; Chioccara, Zeuli, & Novellino, 1986; Grant, Middleton, Plack, & Thomson, 1985). No search of gadusol in fish species from the southern hemisphere has been published to our knowledge.

Gadusols and MAAs are biosynthesised *via* the shikimate pathway which is thought to be restricted to bacteria, algae, plants and fungi. Fish and other marine organisms probably accumulate these compounds by diet acquisition or by symbiotic or bacterial association (Shick & Dunlap, 2002).

The antioxidant capacity of deoxygadusol has been evaluated through the phosphatidylcholine peroxidation inhibition-assay, yielding a larger activity than mycosporine-glycine (Dunlap, Shick, & Yamamoto, 2000). Other assays have demonstrated that redox properties of gadusol are similar to those of ascorbic acid (Plack et al., 1981; Shick & Dunlap, 2002).

In this work, we report on the evaluation of the presence of the natural antioxidant gadusol in mature ovaries of argentine hake (*Merluccius hubbsi*), brazilian sandperch (*Pinguipes brasilianus*) and argentinian sandperch (*Pseudopercis semifasciata*), all from Argentine Sea. In addition, we present the results of the assessment of the antioxidant activity of the isolated compound in aqueous medium, in terms of the hydrophilic chain-breaking antioxidant capacity against the peroxyl radical and the ability to reduce a probe radical-cation.

2. Materials and methods

2.1. Chemicals

For the extraction of gadusol, absolute ethanol, chloroform, acetic and hydrochloric acid, anhydrous sodium acetate, monobasic potassium phosphate and dibasic sodium phosphate (all P.A. grade) were provided by Cicarelli Laboratories (Argentina). Ion-exchange resins DOWEX® 50WX and DOWEX® 1X8 were purchased from Sigma–Aldrich and 96% v/v ethanol was from Porta (Argentina). Solutions for the antioxidant activity determination were prepared in HPLC-grade water (Sintorgan). ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) was from Fluka and potassium persulfate ($K_2S_2O_8$), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one) and ABAP (2,2′-azobis(2-amidinopropane) hydrochloride) were from Aldrich. Buffer solutions at pH 7.2 and 7.4 were prepared with potassium phosphate dibasic (Fluka) and potassium phosphate monobasic (Sigma–Aldrich). All reagents were > 99% pure.

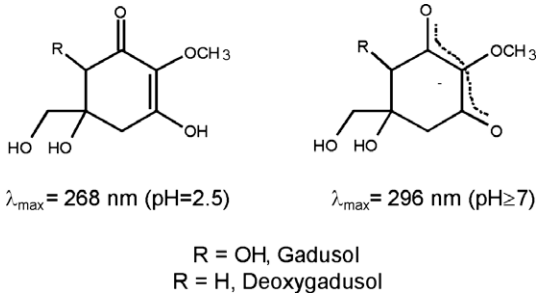

Fig. 1. Chemical structures of gadusol and deoxygadusol.

2.2. Biological material

Mature female gonads from argentine hake (*M. hubbsi*), brazilian sandperch (*P. brasilianus*) and argentinian sandperch (*P. semifasciata*) were used as sources of gadusol. All the specimens were collected from the coast of the Argentine Sea (approximately 30° S to 50° S). Once received, the gonads were stored at −20 °C until processing.

2.3. Extraction of gadusol

The procedure described by Plack et al. (1981) was followed with minor modifications. Unfrozen 50 g samples of the gonad contents (roes devoid of pelt) were homogenised for 2 min with 200 ml of absolute ethanol by means of a top-drive type homogeniser. The homogenate was sonicated for 5–10 min and kept overnight at 6 °C. The mixture was centrifuged at 5000*g* for 10 min and filtered to separate the supernatant. The solid residue was washed with 500 ml of 80% v/v ethanol and re-homogenised with 1 l of this solution by sonication for other 5–10 min. The extracts were once again centrifuged and filtered, and the filtrates were combined and concentrated to *ca.* 50 ml in a pressure-reduced Büchi RE-111 rotary evaporator with water bath at 40 °C. An opalescent-yellow solution was obtained and transferred to a separating funnel. There, the solution was successively washed with 50 ml of absolute ethanol, two lots of 50 ml of chloroform and two lots of 50 ml of bi-distilled water. After shaking, the mixture was kept overnight at room temperature. The lower (organic) phase containing mainly lipids was discarded. The aqueous phase was washed with 100 ml of chloroform and concentrated again to *ca.* 15 ml. The concentrated solution was treated by ion-exchange chromatography on DOWEX® 50WX (8–400, H^+ form) resin (column dimensions: 25 mm i.d. × 100 mm length). A pre-treatment of the resin with HCl 10% v/v was performed to remove impurities. The elution was carried out with bi-distilled water at 5–6 drops per min. The fractions containing gadusol (6 ml) were spectrophotometrically recognised by their maximal absorption at 268 nm (Chioccara et al., 1980; Grant et al., 1985). Reversible shift of the maximum to 296 nm at pH ⩾ 7 was taken into account as an additional criterion of fraction selection (Bandaranayake et al., 1997; Plack et al., 1981). Also reversed phase high-performance liquid chromatography (HPLC) with a mobile phase at pH 4 was carried out on the diluted fractions. The selected fractions were combined, evaporated to dryness and re-dissolved in 2 ml of 0.5 M acetic acid. This solution was applied to a column (9 mm i.d. × 140 mm length) of DOWEX® 1X8 (200–400 mesh, Cl) ion-exchange resin, previously converted to the acetate form by washing with 3 M sodium acetate. The column was balanced and eluted with 0.5 M acetic acid. The fractions containing gadusol were combined and evaporated, and applied to a second column of DOWEX® 1X8 resin after re-dissolution in 2 ml of 0.5 M acetic acid. Fractions of 3 ml were collected and stored at −20 °C until use.

2.4. Instrumental analysis

UV–visible absorbance spectra were recorded on a Shimadzu UV-2001 PC scanning system with 1-cm quartz cuvettes. A Konik KNK-500-A HPLC system with a Konik UVIS-200 absorbance detector and data acquisition software was used together with a reverse

phase column Aqua C-18 (5 μm, 4.6 mm i.d. × 250 mm length, Phenomenex) and alternative aqueous mobile phases of acetic acid 0.01% (pH 4), 0.5 M (pH 2.5) or phosphate buffer (pH 7) at 1 ml min^{-1} flow rate. Detection of gadusol was achieved at the maximal absorption wavelength in the medium.

^1H NMR analysis for structure confirmation of the isolated gadusol was carried out in a FT-NMR Brüker instrument at 200 MHz on *ca.* 2 mM solutions in D_2O. The samples were prepared by previously removing the solvent from the stock solution by freezing and pumping in an Ar atmosphere.

2.5. Determination of antioxidant activity

The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out by following the procedures reported by Naguib (2000). Fluorescein was used as a probe that loses its fluorescence on oxidation by peroxyl radicals. Trolox was taken as a reference antioxidant. The rate of the fluorescence decay decreased in the presence of the antioxidant (gadusol or Trolox) due to the scavenging of the peroxyl radicals. All the solutions were prepared in 0.075 M phosphate buffer at pH 7.2. Fluorescence emission was measured in a 1-cm quartz cuvette on a Spex–Fluoromax spectrofluorometer equipped with a stirrer and a temperature-controlled cell holder at 37 °C. The excitation and emission wavelengths were 495 nm and 520 nm, respectively. Peroxyl radicals were generated by thermal decomposition of ABAP in air-saturated solutions.

The chemical stability of 15 nM fluorescein solutions in the presence of gadusol was checked by recording the fluorescence emission for 20–30 min. Control measurements were also carried out in the absence of antioxidant, probing the thermal stability of the fluorescein solutions.

Different ABAP concentrations were assayed and a final one of 10.22 mM was chosen for a convenient total reaction time.

After addition of ABAP to mixture solutions of fluorescein and the antioxidant at a final concentration between 0.30 and 1.10 μM, the fluorescence intensity was recorded every minute until reaching a value close to zero.

Thus, the peroxyl radical-scavenging activity of gadusol relative to Trolox was calculated according to Eq. (1):

$$\text{Relative ORAC value} = \left[\frac{\text{AUC}_{\text{gadusol}} - \text{AUC}_{\text{blank}}}{\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}}}\right] \times \frac{M_{\text{Trolox}}}{M_{\text{gadusol}}} \quad (1)$$

where AUC stands for the area under the curve of the probe fluorescence decay *vs.* time (Naguib, 2000). The area was evaluated in terms of the values f_t of the fluorescence emission at each time t as

$$\text{AUC} = 1 + \sum f_t/f_i \quad (2)$$

In this expression, f_i is the initial reading of the fluorescence signal.

The ABTS assay, described by Re et al. (1999), has been used with minor modifications. This assay is based on the capacity of a compound to scavenge the $ABTS^{\cdot+}$ radical-cation. The absorbance changes of $ABTS^{\cdot+}$ at 734 nm in presence of the antioxidant were determined spectrophotometrically. The decolouration degree was expressed as TEAC-values (Trolox Equivalent Antioxidant Capacity). The cation $ABTS^{\cdot+}$ was pre-formed by reaction between ABTS and $K_2S_2O_8$ aqueous solutions at final concentrations of 7 mM and 2.45 mM, respectively. The reaction was carried out in the dark overnight at room temperature. $ABTS^{\cdot+}$ was diluted with buffer solution (pH 7.4) to a final absorbance of 0.6–0.7 at λ = 734 nm. Appropriate blanks were used as controls in order to correct dilution effects. The stock solution was kept at 6 °C during the experiment in order to minimise the decomposition of $ABTS^{\cdot+}$.

For each experiment, aliquots of the antioxidant were added to solutions of $ABTS^{\cdot+}$ to get a final concentration between 2 and 15 μM. All experiments were carried out in triplicate at 25 °C on thermostatised bath. The TEAC-values were calculated as function of time, at selected points along the reaction (1, 4 and 6 min), from the ratio between the slopes of the percentage of absorbance inhibition *vs.* antioxidant concentration for gadusol and Trolox.

3. Results and discussion

3.1. Gadusol content

The presence of gadusol was examined on samples of mature ovaries from argentine hake (*M. hubbsi*), brazilian sandperch (*P. brasilianus*) and argentinian sandperch (*P. semifasciata*). The crude (ethanolic) extracts from brazilian and argentinian sandpearchs showed the characteristic bathochromic shift of gadusol absorption spectrum upon increase of pH (Chioccara et al., 1980) (Fig. 2). Unlike these, crude extracts from hake roes did not show that behaviour.

The reductive character of the purified samples from *P. brasilianus* and from *P. semifasciata* (sandpearchs) was demonstrated by the bleaching of aqueous bromine or triyodide solutions as qualitatively observed by spectrophotometric analysis (Plack et al., 1981).

HPLC analysis of the fractions from the sandpearchs, after the complete separation routine, yielded both a unique peak with *ca.* 6 min retention time under the pH 2.5 mobile phase and one peak with *ca.* 4 min retention time under the pH 7 buffered phase. The shift of retention times with the change of pH of the mobile phase is consistent with the presence of gadusols in the neutral and enolate forms respectively.

The ^1H NMR signals for the purified samples in D_2O were: 4.18 (1H, s, H-6); 3.61 (1H, d, J = 12 Hz, H-α7); 3.45 (1H, d, J = 12 Hz, H-β7); 3.49 (3H, s, H-8); 2.74 (1H, d, J = 18 Hz, H-α4); 2.42 (1H, d, J = 18 Hz, H-β4). These results agree with those reported by Bandaranayake et al. (1997) and Chioccara et al. (1980) confirming the structure of gadusol. The presence of both gadusol and deoxygadusol in a ratio 4:1 in marine organisms has been reported by Grant et al., 1985 and Chioccara et al., 1980. However, our analysis indicates that deoxygadusol is at least below detectable levels in the extracts.

The content of gadusol in the extracts was estimated on the basis of the absorbance at 268 nm and at pH 2.5 and the molar absorption coefficient ε_{268} = 12,400 $M^{-1}cm^{-1}$ (Plack et al., 1981). The results are summarised in Table 1. The yields of *P. brasilianus* and *P. semifasciata* are four- to sixfold larger than those obtained by Chioccara et al. (1980) in species of the same order *Perciformes*.

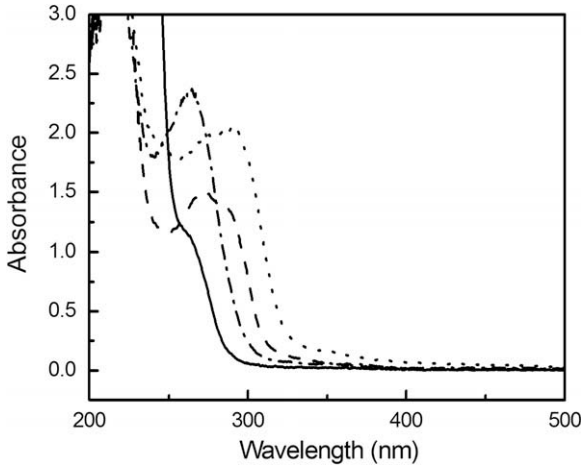

Fig. 2. Absorption spectra of aqueous ethanolic dilutions of the extracts from argentinian sandperch in acid (-·-·-) and basic (····) media and from brazilian sandperch in acid (—) and basic (- - -) media.

Table 1
Concentration of gadusol in roes of some marine fish from Argentine sea.

Source	Gadusol content (mg/kg wet tissue)
Argentinian sandperch (*Pseudopercis semifasciata*)	245
Brazilian sandperch (*Pinquipes brasilianus*)	175
Argentine hake (*Merluccius hubbsi*)	0

However, no gadusol was found in *M. Hubbsi* within the detection limits of the analytical techniques. Interestingly, the last species belongs to the order *Gadiformes* like the Atlantic cod (*Gadus morhua L.*), found in the northern hemisphere with *ca.* 4.3 mg gadusol/g (Plack et al., 1981). This result suggests that the accumulation of gadusol in fish roes may be correlated with the habitat and food availability of the animal rather than with the capability of *de novo* synthesis of the compound.

Although the processed samples consisted of mature roes, the exact maturation level was not determined. According to the studies of Plack et al. (1981), the quantity of gadusol increased almost linearly during the course of egg development in the ovaries, whereas it was at the limit of detectability in other tissues. These facts point to a functional role of gadusol in embryonic development probably associated to the antioxidant properties of the compound.

3.2. Antioxidant activity

The assessment of the antioxidant activity of gadusol was carried out by the ORAC and ABTS assays.

Fig. 3 shows the results for the ORAC assay as fluorescence time profiles of the probe fluorescein in the absence or presence of the antioxidant compounds (gadusol or Trolox as a reference) at comparable concentrations. The signal from the fluorescent probe alone was taken as a control measurement. Also the chemical stability of the mixture of fluorescein and gadusol was verified (signals not shown).

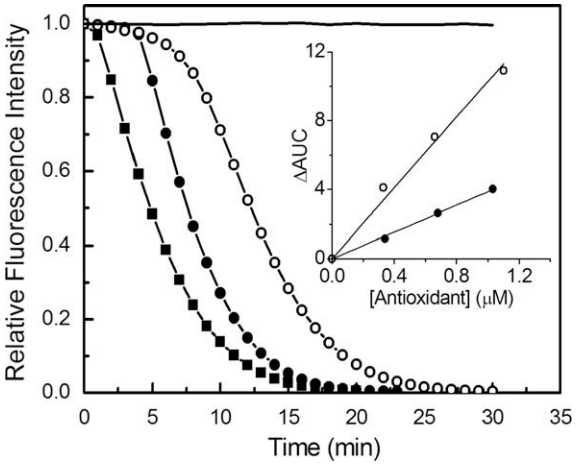

Fig. 3. Time profiles of the fluorescence emission at 520 nm of the probe fluorescein for the ORAC assay. Black squares show the fluorescence decay in the presence of ABAP without antioxidant (blank experiment). The full line denotes the control experiment that accounts for the thermal stability of fluorescein solutions at 37 °C. Full and open circles correspond to the fluorescence signals in the presence of 0.68 μM Trolox and 0.66 μM gadusol, respectively. The inset shows the linear correlation between the net area under the curves ($\Delta AUC = AUC_{Antiox} - AUC_{Blank}$) and the antioxidant concentration with regression coefficients of $r = 0.9990$ and $r = 0.9961$ for Trolox and gadusol, respectively.

It is evident from Fig. 3 that gadusol shows greater antioxidant activity than the reference compound Trolox. A complete protection of the fluorescent probe is manifested by the presence of a lag phase, which indicates that gadusol reacts with peroxyl radicals much more rapidly than fluorescein. Once the antioxidant is completely consumed, the decay of the signal is similar to that of the blank. Furthermore, kinetics modelling of the ORAC assay recently carried out suggests that the lag phase for loss of fluorescence results from an equilibrium between antioxidant and fluorescein radicals (Bisby, Brooke, & Navaratnam, 2008).

The effect of the antioxidant concentration on the ORAC values can be explored in the inset of Fig. 3. ΔAUC, defined as the difference ($AUC_{antiox} - AUC_{blank}$), increases linearly with gadusol content. An analogous behaviour was verified for ascorbic acid, which was explored for comparative purposes. Although our results for the relative antioxidant activity were slightly lower than those already reported (Naguib, 2000), the linearity between ΔAUC values and concentration was also observed ($r = 0.9998$).

The relative antioxidant activity measured by ORAC determinations was calculated according to Eq. (1).

The ABTS assay measures the ability of a compound to scavenge the $ABTS^{\cdot+}$ radical-cation. The absorbance of the radical-cation alone at 25 °C proved its stability in the media within a decrease of 9% after 30 min.

The effect of the addition of gadusol on the decay of $ABTS^{\cdot+}$ absorbance (not shown) can be separated in two regions: a fast one within the first minute and a slower one at larger times. The profile is very different for Trolox, with the reaction complete in less than 10 s, in agreement with previous results from Campos and Lissi (1997). This behaviour has also been observed for glutathione, cyanidin, quercetin and α-tocopherol (Re et al., 1999) and may be ascribed to a complex reaction mechanism that implies the contribution of a product of the first oxidation step further reacting with the radical at a lower rate (Arts, Dallinga, Voss, Haenen, & Bast, 2003). Thus, some authors suggest distinguishing the activity as fast, slow or total scavenging (Johnston et al., 2006). Consistently, the activity has been explored in terms of TEAC for three selected reaction times (Table 2) as proposed by Re et al. (1999). The TEAC-values were obtained as the ratio of the slopes of the curves for gadusol at each of the times and the respective slopes for Trolox. The explored concentration ranges for Trolox and gadusol were similar. The inhibition of the $ABTS^{\cdot+}$ absorbance *vs.* gadusol concentration at different times is shown in Fig. 4a.

On the other hand, the degree of inhibition depends on gadusol concentration. Fig. 4 a and b show that straight lines fit the experimental points at concentrations lower than *ca.* 6 μM for gadusol. These linear fits were used for the calculation of the TEAC-values shown in Table 2. This dependence might be explained in terms of the change of the stoichiometric factor for the reaction between the antioxidant and the radical due to secondary reactions of the intermediates (Campos and Lissi (1997)).

The results for the ORAC determination in Table 2 reveal that gadusol activity is *ca.* sixfold that for ascorbic acid, although lower than that of the strong flavonoid-antioxidant quercetin (Naguib, 2000). However, the ABTS assay yielded similar antioxidant activities for gadusol and ascorbic acid, whilst they keep lower than quercetin (Re et al., 1999).

Table 2
Antioxidant activity of gadusol determined by ORAC and ABTS assays.

ORAC	ABTS		
Relative antioxidant activity[a]	TEAC (1 min)	TEAC (4 min)	TEAC (6 min)
2.6	0.96	1.27	1.40

[a] Calculated as Trolox equivalents according to Eq. (1).

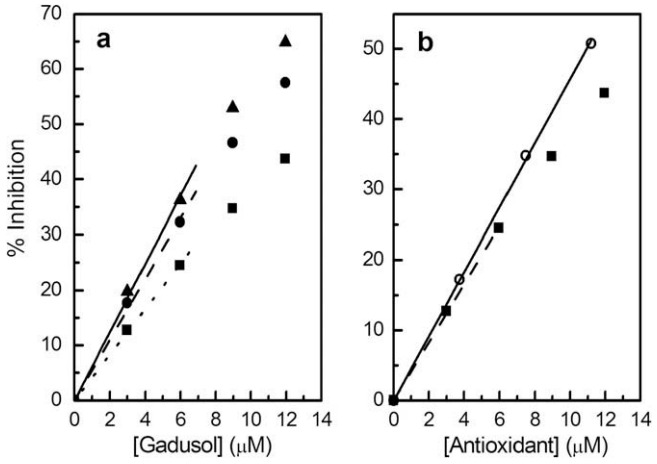

Fig. 4. (a) Inhibition of $ABTS^{\cdot+}$ absorbance as a function of gadusol concentration for different time intervals: 1 min (squares), 4 min (circles) and 6 min (triangles). (b) Comparison of the inhibition of $ABTS^{\cdot+}$ absorbance after 1 min for gadusol (squares) and Trolox (open circles).

The reasons of the latter differences are to be found in the nature of each reaction mechanism. On one side, the ORAC assay is based on competitive kinetics that provides a direct measure of hydrophilic chain-breaking antioxidant capacity against a peroxyl radical through a hydrogen atom transfer mechanism. The ABTS assay instead may operate also or exclusively through an electron transfer mechanism, which implicates a reduction reaction of a radical species as an end point indicator (Huang, Ou, & Prior, 2005). Besides, an ORAC assay provides a more complete measure since it combines the fraction of quenched radical with the total inhibition time, warranting that all the antioxidant has been consumed. On the contrary, an ABTS assay usually underestimates the antioxidant capacity since it requires longer reaction times (at least 30 min) for completion through the successive steps that involve the intermediate products, once the fast scavenging period has taken place.

The similar values yielded by the ABTS determination for gadusol and ascorbic acid can be explained by comparable redox potentials of the species, a property that has been directly related with the type of reaction assessed (Huang et al., 2005).

Finally, the results for gadusol from both assays (Table 2) are in a ratio of *ca.* 2, suggesting that the compound is more efficient as a peroxyl radical scavenger than as a simple reducing agent. This is particularly relevant considering the key role played by peroxyl radicals in the unwanted lipid oxidation in food and biological systems.

Further work to determine the redox potential of gadusol and the detailed kinetic mechanisms that support the antioxidant capacity here evaluated is underway.

4. Conclusion

Results from this study show that the presence of the metabolite gadusol is significant in gonad tissues of brazilian sandperch (*P. brasilianus*) and argentinian sandperch (*P. semifasciata*) from Argentine Sea. However gadusol was below detectable levels in ovaries from argentine hake (*M. hubbsi*).

The quantitative evaluation of the antioxidant potential of purified gadusol from fish roe extracts by ORAC and ABTS assays confirms that the natural compound is comparable to ascorbic acid towards reductive reactions of radicals and that it bears a strong ability to break chain reactions carried by peroxyl radicals.

These results support that roes from these fish species, and probably from other widely consumed sort of fishes that should be evaluated in the future, might be considered as appropriate sources of antioxidants for human diet. Further, potential applications of natural bioproducts such as gadusol in the formulation of nutraceutical and medical products need to be studied.

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