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Antioxidant effect of quinoline derivatives containing or not selenium: Relationship with antinociceptive action quinolines are antioxidant and antinociceptive

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ABSTRACT

The present study investigated the antioxidant effect of a new class of quinoline derivatives (**a-d**) on assays *in vitro*. Lipid peroxidation, thiol peroxidase-like and free radical scavenging activities were determined to evaluate antioxidant activity of compounds. Thiol oxidase-like and δ-aminolevulinate dehydratase activities were performed as a toxicological parameter. A second objective of this study was to evaluate the *in vivo* antinociceptive effect of the compound with better antioxidant effect and without toxic effects in a model of nociception induced by formalin in mice. In liver, at 100 µM, compound **a** reduced the lipid peroxidation to the control levels, while compounds **c** and **d** partially reduced it. In brain, only compound **d** partially reduced the lipid peroxidation at 50 and 100 µM. Compound **b** did not have an effect on the lipid peroxidation. Thiol peroxidase-like and free radical scavenging activities are not involved in the antioxidant mechanisms of these compounds. Compounds did not present thiol oxidase-like activity and effect on the δ-aminolevulinate dehydratase. *In vivo* experiments showed that compound **a** caused an inhibition of licking time in the first and second phases, and edema formation induced by formalin. In conclusion, quinoline derivative without selenium presented better *in vitro* antioxidant effect and *in vivo* antinociceptive activity.

Key words: antinociceptive, antiedematogenic, anti-inflammatory, antioxidant, quinoline.

INTRODUCTION

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidant systems. In fact it is characterized by an increase in the reactive species (RS) production and/or a decrease in the antioxidant defenses. RS are a normal product of cellular metabolism, and they are involved in important biological functions. However, excessive production of RS may generate oxidative stress, causing tissue damage. Furthermore, several diseases are related to oxidative stress, such as such as cardiovascular and neurodegenerative diseases,

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cancer, atherosclerosis, rheumatoid arthritis, hypertension, ischemia and diabetes mellitus (Souza et al. 2009, Rajendran et al. 2014).

In this sense it is important to use antioxidant molecules that decrease oxidative stress, reducing the incidence of various diseases (Gay et al. 2010, Maes et al. 2011, Gupta et al. 2014). It is therefore important to highlight organoselenium compounds, which exhibit different pharmacological properties, such as antioxidant action (Nogueira and Rocha 2010, Luchese et al. 2012, Sancineto et al. 2016). Parallel to organoselenium compounds are quinoline derivatives. Quinolines are synthetic or natural heterocyclic compounds with interesting biological activities (Kouznetsov et al. 2005, Manjunatha et al. 2013).

Although several research groups have studied the mechanisms involved in the pharmacology of organochalcogens (Seng and Tiekink 2012, Ibrahim et al. 2012), the study of a quinoline derivative containing selenium could be an alternative to search for new compounds with antioxidant properties for the treatment of diseases related to oxidative stress. In addition, oxidative stress has been proposed as a mechanism involved in the pathophysiology of inflammation and pain (Abdel-Wahab and Salama 2011, Kubera et al. 2011). Pain and inflammation are a major clinical problem, and several undesirable side effects caused by the use of analgesic and anti-inflammatory agents occur (Sheeba and Asha 2009, Riedel et al. 2015). In view of this, the development of new drugs with antinociceptive effects for the control of several painful conditions would be very useful as a therapeutic source.

Our research group has described the synthesis and pharmacological properties of quinolines and their derivatives, demonstrating potential antioxidant (Saraiva et al. 2016, Savegnago et al. 2013) and antinociceptive effects (Wilhelm et al. 2014, Pinz et al. 2016). Following our longstanding interest in pharmacological and toxicological properties of quinoline and its derivatives, this study investigated the *in vitro* antioxidant and toxicological effects of quinoline derivative containing or not selenium. Considering the results obtained *in vitro*, a second objective of this study was to investigate the *in vivo* antinociceptive activity of compound with better antioxidant effect and without toxic effects in a model of nociception induced by formalin in mice.

MATERIALS AND METHODS

CHEMICALS

2,4-diphenylquinoline (Figure 1a), 2,4-diphenyl-3-(phenylselanyl)quinoline (Figure 1b), 6-chloro-2,4-diphenyl-3-(phenylselanyl)quinoline (Figure 1c), 6-nitro-2,4-diphenyl-3-(phenylselanyl) quinoline (Figure 1d) were designated **a**, **b**, **c** and **d**, respectively. These compounds were prepared according to the method found in the literature (Stein et al. 2015) and used at different concentrations (μM) and doses (mg/kg). Analysis of the ^{1}H nuclear magnetic resonance and ^{13}C nuclear magnetic resonance spectra showed that all compounds synthesized exhibited analytical and spectroscopic data in full agreement with their assigned structures. Compounds **a-d** were dissolved in dimethyl sulfoxide to *in vitro* (initial concentration of 50 mM) assays and in canola oil for *in vivo* (initial concentration of 36 mM) experiments. Formalin was dissolved in saline (sodium chloride 0.9%).

ANIMALS

The experiments were conducted using male adult Swiss mice (25–35 g). The animals were kept in a separate animal room, in a 12 h light/dark cycle (with lights on at 6:00 a.m.), at a room temperature of 22 ± 2ºC, with free access to food and water. The experiments were performed according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Pelotas, Brazil (CEEA 1987 and

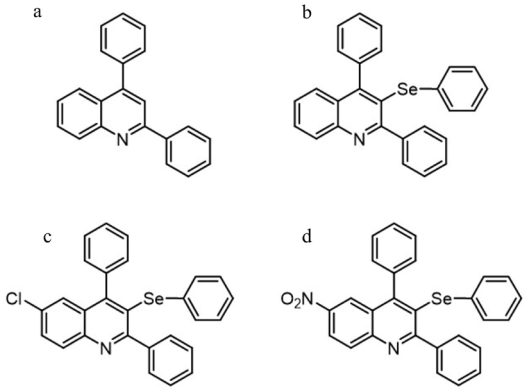

Figure 1 - Chemical structure of quinoline derivatives. (a) 2,4-diphenylquinoline; (b) 2,4-diphenyl-3-(phenylselanyl) quinoline; (c) 6-chloro-2,4-diphenyl-3-(phenylselanyl) quinoline; (d) 6-nitro-2,4-diphenyl-3-(phenylselanyl) quinoline.

1287-2016). The number of animals (104 animals) and intensities of noxious stimuli (20 µl of 2.5 % formalin administered in the animals' paws used were the minimum necessary to demonstrate the consistent effects of the drug treatments. For *in vivo* experiments, 7 to 8 animals were used per group. For lipid peroxidation assays and δ-aminolevulinate dehydratase (δ-ALA-D) activity, the experiments were performed 3 times and 1 animal was used for each experiment.

IN VITRO ASSAYS

Tissue preparation

Mice were euthanized by inhalation of isoflurane. Brain and liver tissues were rapidly dissected, weighed, placed on ice, homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5 for brains, 1/10 for livers, w/v) and centrifuged at 900 *x* g at 4°C for 10 minutes. Supernatant was used for lipid peroxidation analysis.

Thiobarbituric acid reactive species (TBARS) levels

TBARS production was performed as a measure of lipid peroxidation (Ohkawa et al. 1979). This assay was carried out to determine if compounds (**a-d**) protected against lipid peroxidation induced by sodium nitroprusside (SNP) in mouse brain and liver homogenates, indicating the antioxidant effect of compounds. An aliquot (200 µl for brain, 100 µl for liver) of supernatant was added to the reaction: SNP (50 µM), compounds (**a-d**) (1–100 µM) and 50 mM Tris–HCl (30 µl). Afterward the mixture was pre-incubated for 1 hour at 37ºC. The reaction product was determined using 500 µl thiobarbituric acid, 200 µl sodium dodecyl sulphate (SDS), and 500 µl acetic acid and incubated at 95ºC for 2 hours. Absorbance was measured at 532 nm in a spectrophotometer. Results were reported as nmol malondialdehyde (MDA) /mg protein.

2,2'-Diphenyl -1-picrylhydrazyl (DPPH) radicals scavenging activity

The scavenging effect of DPPH radicals was performed to determine the possible mechanism by which the compounds (**a-d**) exhibit antioxidant property. In the DPPH assay, the reduction of DPPH radical by antioxidants could be for the donation of hydrogen atom to a free radical to remove the extra electron (which is responsible for the activity of free radicals) (Thaiponga et al. 2006). A DPPH stable radical assay was performed in accordance with Choi et al. (2002). Briefly, DPPH was added to a medium containing compounds (**a-d**) at different concentrations (1-100 µM). The medium was incubated for 30 minutes at room temperature. The decrease in absorbance was measured at 517 nm, which depicted the scavenging activity of compounds (**a-d**) against DPPH. Ascorbic acid (50 µM) was used as a positive control. The values were expressed in percentage of blank: DPPH radicals

scavenging activity = (absorbance of sample x 100) / absorbance of blank.

2,2'-Azino bis-3-ethylbenzothiazoline-6 sulfonic acid (ABTS) radicals scavenging activity

The scavenging effect of ABTS radicals was performed to determine the possible mechanism by which compounds (**a-d**) exhibit antioxidant property. ABTS is a protonated radical and it is used for evaluating the scavenger activity of proton radicals (Thaiponga et al. 2006). The ABST radical assay was performed in accordance with Re et al. (1999). In short, ABTS was added to a medium containing compounds (**a-d**) at different concentrations (1-100 μM). The medium was incubated for 30 minutes at room temperature. The decrease in absorbance was measured at 734 nm, which depicted the scavenging activity of compounds (**a-d**) against ABTS. Ascorbic acid (50 μM) was used as a positive control. The values were expressed in percentage of blank: ABTS radicals scavenging activity = (absorbance of sample x 100) / absorbance of blank.

Thiol peroxidase-like activity

The antioxidant action of quinoline derivatives could be explained by their ability to mimic the activity of the thiol peroxidase enzyme. Indeed, several organoselenium compounds have a catalytic activity similar to that of the thiol peroxidase enzyme, reducing peroxides at the expense of thiol compounds (Mugesh and Singh, 2000, Nogueira et al. 2004).

Thiol peroxidase-like activity was carried out to determine the possible mechanism by which compounds (**a-d**) (1–100 μM) have antioxidant properties. Free-SH groups were determined according to Ellman (1959). Compounds (**a-d**) were incubated in the medium containing reduced glutathione (GSH) (1.0 mM) with and without H_2O_2 (0.3 mM) and checked after 120 min, for the amount of GSH. The values are expressed in percentage of control.

Thiol oxidase-like activity

Thiol oxidase activity determines the pro-oxidant property of compounds. Thiol oxidation was determined with 50 mM Tris–HCl, pH 7.5, compounds (**a-d**) (1–100 μM) and addition of 1.0 mM GSH. Aliquots were checked for the amount of thiols groups. Free thiols groups were determined according to Ellman (1959). The values are expressed in percentage of control.

δ -ALA-D activity

δ-ALA-D activity has been reported as sensitive to pro-oxidant compounds and heavy metals. δ-ALA-D activity was used as a toxicity marker. δ-ALA-D activity was assayed by the method of Sassa (1982) with some modifications. The principle of this method is based on the enzyme incubation with excess of δ-aminolevulinic acid (δ-ALA). An aliquot (200 μl) of supernatant was pre-incubated for 10 minutes at 37ºC in the presence or absence of compounds (**a-d**) at different concentrations (1-100 μM). Enzymatic reaction was initiated by adding the substrate (δ-ALA, 12 mM). The incubation was carried out for 3 hours at 37ºC. The reaction was stopped by adding trichloroacetic acid solution (10 % TCA) with 10 mM $HgCl_2$. The porphobilinogen, which is formed during the incubation period, was mixed with modified Ehrlich's reagent, and the color developed was measured spectrophotometrically (555 nm) against a blank. Results were expressed as nmol porphobilinogen (PBG)/h/mg protein.

Protein quantification

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

IN VIVO EXPERIMENTS

Formalin-induced nociception

The formalin test was carried out as described by Hunskaar and Hole (1987). Mice were pretreated with compound **a** (1, 10, 50 mg/kg, per oral route (p.o.)), meloxicam (50 mg/kg, p.o.) or an appropriate vehicle. After 30 minutes, the animals received the intraplantar (i.pl.) administration of formalin (2.5 %, v/v; 20 µl/paw) in the right hind paw and saline solution (0.9 %, w/v; 20 µl/paw) in the left paw. The animals were individually placed in an acrylic box and duration of paw licking was recorded at 0-5 min (first phase) and 15-30 min (second phase). The first phase is regarded as a neurogenic mechanism and the second phase as an inflammatory mechanism. After the period of observation, mice were sacrificed and the edema was measured. Paws were removed and weighed. The paw edema was measured by comparing the difference between the weight of the formalin-injected paw and the weight of the contralateral paw (saline-treated paw).

STATISTICAL ANALYSIS

Data were expressed as mean ± S.E.M. Statistical analysis was performed using a one-way ANOVA followed by the Newman-Keuls test when appropriate. Values of $p < 0.05$ were considered statistically significant. Maximal inhibition (I_{max} - maximum percentage by which an inhibitor reduced a response) was calculated at the most effective concentration used.

RESULTS

EFFECT OF COMPOUNDS **A-D** ON TBARS LEVELS

In the brain, compound **d** partially reduced the lipid peroxidation induced by SNP at concentrations equal to or greater than 50 µM, while compounds **a**, **b** and **c** did not have any effect on the lipid peroxidation (Figure 2a). I_{max} was in the following order: **d** (26 %) > **a** (12 %) > **b** (10 %) > **c** (1 %).

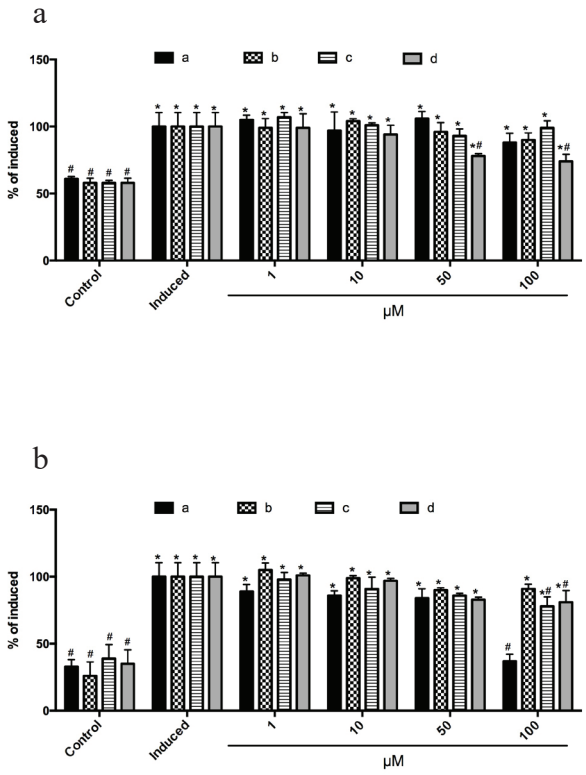

Figure 2 - Effect of compounds **a - d** on lipid peroxidation induced by SNP in the brain **(a)** and liver **(b)** of mice. Data are reported as mean ± S.E.M. Statistical analysis was performed by One-way ANOVA followed by Newman-Keuls' test. (*) denotes $P < 0.05$ when compared to the control group. (#) denotes $P < 0.05$ when compared to the induced group.

In the liver, compound **a** was effective in reducing TBARS levels at a concentration equal to 100 µM (Figure 2b). Compounds **c** and **d**, at a concentration equal to 100 µM, partially reduced the lipid peroxidation induced by SNP, while compound **b** did not have any effect (Figure 2b). I_{max} was in the following order: **a** (63 %) > **c** (22 %) > **d** (19 %) > **b** (9 %).

EFFECT OF COMPOUNDS **A-D** ON DPPH RADICAL SCAVENGING ACTIVITY

Compounds **a-d**, at all concentrations tested, did not present DPPH radical scavenging activity (data not shown). Ascorbic acid, a positive control, at

the concentration of 50 µM showed DPPH radical scavenging activity (data not shown).

EFFECT OF COMPOUNDS **A-D** ON ABTS RADICAL SCAVENGING ACTIVITY

Compounds **a-d**, at all concentrations tested, did not present ABTS radical scavenging activity (data not shown). Ascorbic acid, a positive control, at the concentration of 50 µM showed ABTS radical scavenging activity (data not shown).

EFFECT OF COMPOUNDS **A-D** ON THIOL PEROXIDASE-LIKE ACTIVITY

Compounds **a-d** did not present thiol peroxidase-like activity (data not shown).

EFFECT OF COMPOUNDS **A-D** ON THIOL OXIDASE-LIKE ACTIVITY

Compounds **a-d** did not present thiol oxidase-like activity (data not shown).

EFFECT OF COMPOUNDS A-D ON Δ-ALA-D ACTIVITY

Compounds **a-d**, at all concentrations tested, did not have an effect on δ-ALA-D activity in the brain and liver of mice (data not shown).

FORMALIN-INDUCED NOCICEPTION

Compound **a** showed antinociceptive activity in the first (ANOVA: $F_{4,33} = 3.651$, $p < 0.05$) and the second (ANOVA: $F_{4,33} = 8.685$, $p < 0.0001$) phases of the formalin test, when compared to the control group (Figure 3a and 3b, respectively).

In the first phase, the animals pretreated with compound **a**, at the doses of 1, 10 and 50 mg/kg, reduced the licking time by 50, 67 and 57 % ($p < 0.05$), respectively (Figure 3a). Meloxicam (50 mg/kg), a reference drug, reduced the licking time (53 %) induced by formalin in the first phase ($p < 0.05$) (Figure 3a).

In the second phase, compound **a**, at the doses of 1, 10 and 50 mg/kg, reduced the licking time

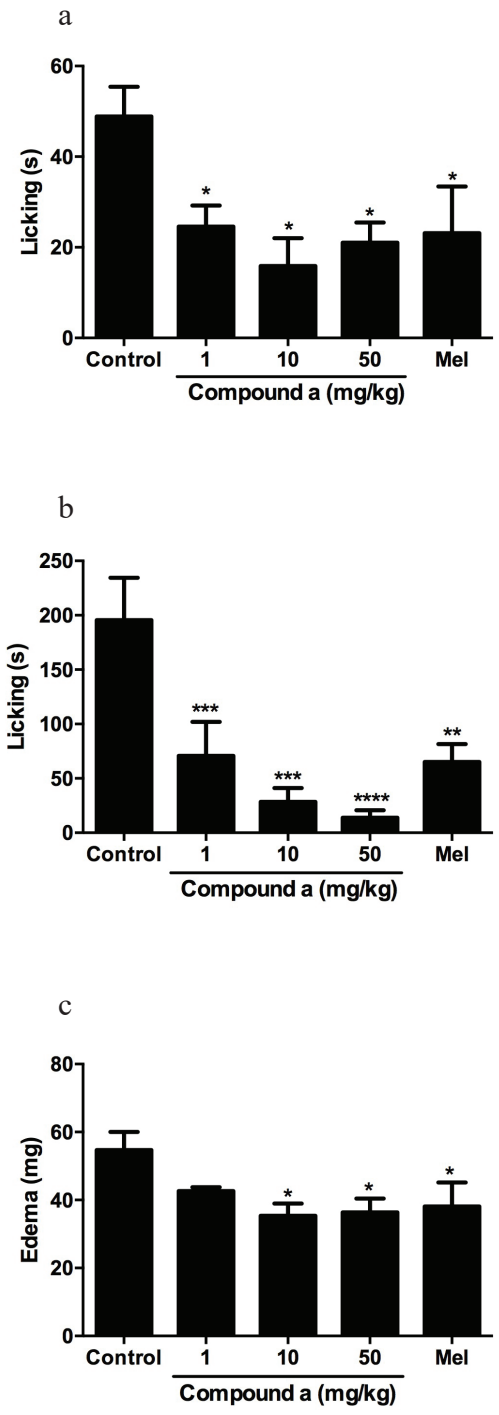

Figure 3 - Effects of compound **a** on formalin-induced nociception. Inhibition of the formalin-induced licking (s) response **(a)** in the first and **(b)** second phases, and **(c)** edema (mg). Data are reported as mean ± S.E.M of 7 to 8 animals per group. Statistical analysis was performed by One-way ANOVA followed by Newman-Keuls' test. (*) denotes $P < 0.05$, (**) denotes $P < 0.01$ and (***) denotes $P < 0.001$ when compared to the control group.

by 64 % ($p < 0.001$), 85 % ($p < 0.001$) and 93 % ($p < 0.0001$), respectively, when compared to the control group (Figure 3b). Meloxicam (50 mg/kg) decreased the licking time by 67 % ($p < 0.01$) (Figure 3b).

Compound **a** markedly reduced the formalin-induced paw edema formation (ANOVA: $F(4,33) = 2.999$, $p < 0.05$) by 35 % (10 mg/kg; $p < 0.05$) and 33 % (50 mg/kg; $p < 0.05$), when compared to the control group (Figure 3c). Compound **a** at a dose of 1 mg/kg did not have any effect in reducing paw edema formation induced by formalin ($p > 0.05$) (Figure 3c). Meloxicam (50 mg/kg) reduced the formalin-induced paw edema formation by 30% ($p < 0.05$) (Figure 3c).

DISCUSSION

The present study demonstrated, for the first time, that a new class of quinoline derivatives containing (compound **c** and **d**) or not selenium (compound **a**) presented *in vitro* antioxidant activity in brain and liver, respectively. In addition, thiol peroxidase-like activity, DPPH and ABTS radical-scavenging activities are not involved in the possible antioxidant mechanism of these compounds. Compounds did not present a thiol-group oxidant effect, as demonstrated by thiol oxidase-like and δ-ALA-D activities. *In vivo* experiments showed that compound **a** presented antinociceptive and antiedematogenic effects in the formalin test.

A close inspection of the results revealed that the antioxidant activity of quinoline derivatives containing (compound **c** and **d**) or not selenium (compound **a**) against lipid peroxidation induced by SNP depended on the chemical structure and analyzed tissue. Compound **a**, a quinoline derivative without selenium, exhibited the highest antioxidant activity in the TBARS assay, since it was the only quinoline derivative that completely restored the TBARS content to control levels. Moreover, compound **a** had a higher I_{max}, indicating the higher effectiveness of this compound when compared to the other compounds (**b**-**d**). Compound **a** has the nitrogen atom in the aromatic ring and it forms a radical which contributes to its antioxidant action.

Since compound **a** has an antioxidant effect, substitutions at specific points in the quinoline system were made with the aim of increasing this activity. Regarding the chemical structure of compounds **b**-**d**, all of them presented the phenylselanyl group and different substituents, H (**b**), Cl (**c**) and NO_2 (**d**), bounded to the aromatic ring of the quinoline system. In the liver, the results demonstrated that compounds **c** and **d**, with Cl and NO_2 as substituent, respectively, had a better antioxidant effect in the TBARS assay than compound **b**, but this effect was inferior to that of compound **a**. In the brain, only compound **d** partially reduced the lipid peroxidation. Evidence has shown that the introduction of selenium atom may improve the biological activity of compounds (Arteel and Sies 2001, Rossato et al. 2002). However, in the present study, we demonstrated that the insertion of a phenylselanyl group worsened the antioxidant effect of compound **a** in the liver, but improved it in the brain, demonstrating that antioxidant action depended on the analyzed tissue.

Moreover, compounds **c** and **d** are quinoline derivatives with an electron withdrawing group (Cl and NO_2, respectively) into the aryl group of the quinoline system, while compound **b** is a quinoline derivative with a neutral group (H) in the aryl group of the quinoline system. These results suggest that the introduction of an electron withdrawing into the aromatic ring of quinoline system influences the antioxidant effect of quinoline derivatives. In addition, the data from the literature have evidenced that the introduction of electron withdrawing groups into the organochalcogen compounds has an important role in establishing their application as antioxidants (Prigol et al. 2009, Luchese et al. 2012).

Radicals DPPH and ABTS have been used to determine the scavenger mechanism of antioxidant compounds (Govindappa et al. 2011, Hamsar et al. 2011, Silva et al. 2015). Our results demonstrated that compounds **a-d** did not present DPPH and ABTS radical-scavenger activities, suggesting that radical-scavenger activity is not the mechanism by which compounds displayed an antioxidant effect. In fact, other studies showed that organoselenium compounds or quinoline derivatives with important antioxidant properties did not present DPPH and ABTS radical-scavenger activities (Luchese and Nogueira 2010, Luchese et al. 2012, Savegnago et al. 2013).

The antioxidant action of compounds **a-d** could be explained by their ability to mimic the activity of thiol peroxidase. In this study, we demonstrated that compounds **a-d** did not demonstrate thiol peroxidase-like activity. The findings of this study clearly indicated that the antioxidant effect of compounds **a-d** is not related to the thiol oxidase-like activity. Moreover, it is difficult to clearly explain why these compounds did not show enzyme-like activity. Accordingly, other studies have revealed that some organochalcogen compounds with antioxidant activity did not exhibit thiol peroxidase-like activity (Souza et al. 2009, Gay et al. 2010).

Even though compounds have an antioxidant effect, it is important to study the toxicity of these compounds. The quinoline derivatives containing or not selenium did not present thiol oxidase-like activity and did not inhibit δ-ALA-D activity *in vitro*, demonstrating a poor potential toxicity of these compounds. In fact, thiol-groups oxidation is an estimation of the cytotoxicity of molecules (Prigol et al. 2012). Some studies have described that the toxicity of organoselenium compounds could be associated with δ-ALA-D activity inhibition. δ-ALA-D is a sulfhydryl-containing enzyme, which can be inhibited, in different pro-oxidant situations; this enzyme can be used as a marker of toxicity (Bechara 1996, Pinton et al. 2010, Rocha et al. 2012). This is a satisfactory result since most organochalcogen compounds showed activity to oxidize thiol groups (Nogueira et al. 2004, Luchese et al. 2007, Gay et al. 2010).

In vitro antioxidant assays prompted the choice of compound **a** for the evaluation of antinociceptive, anti-inflammatory and antiedematogenic activities in the formalin test. In the TBARS assays, compound **a** had higher I_{max}. This parameter indicates the effectiveness of compounds. Thus, compound **a** had higher effectiveness than other compounds in the TBARS assays. Moreover, compound **a** exhibited the highest antioxidant activity in the TBARS assay, since it was the only quinoline derivative that completely restored the TBARS content to control levels.

The results of this study showed that compound **a**, a quinoline derivative without selenium, reduced the nociceptive behavior in the first (nociceptive) and second (inflammatory) phases and it presented an antiedematogenic effect in reducing the paw edema formation in the formalin test. These results reinforce the antinociceptive, anti-inflammatory and antiedematogenic potential of compound **a**. The formalin test has long been used as a screening tool for the assessment of antinociceptive property of new agents and it is considered a reliable model of tonic pain of the inflammatory type (Pavin et al. 2011, Lima et al. 2013, Sari et al. 2014). The formaldehyde induces damage in tissues to imitate acute post lesion pain in humans with a biphasic response (Bonjardim et al. 2011). In the acute phase, called neurogenic, nociception is caused by direct activation of the nociceptive fibers, which release substance P, glutamate, and bradykinin, among other pain mediators (Luccarini et al. 2006). The late phase, which is inflammatory, is mediated by the release of inflammatory mediators, such as nitric oxide, excitatory amino acids (aspartate and glutamate), prostacyclins, prostaglandins (PGE2), and leukotrienes, which will later stimulate the

spinal cord and cause pain (Bonjardim et al. 2011, Venancio et al. 2011). In accordance, literature data have indicated that quinoline derivative compounds (present important) important antinociceptive activity (Nogueira et al. 2003, Pavin et al. 2011, Sari et al. 2014, Pinz et al. 2016).

In conclusion, the results of this investigation demonstrated the *in vitro* antioxidant effect of quinoline derivatives containing or not selenium against lipid peroxidation. However, antioxidant mechanisms of compounds were not found, ruling out thiol peroxidase-like activity, and DPPH and ABTS radical-scavenger activities. Moreover, we demonstrated the antinociceptive, anti-inflammatory and antiedematogenic effects of compound **a**, the best antioxidant compound. Further studies are necessary to demonstrate other pharmacological properties of these compounds *in vivo*, as well as the mechanisms involved in the antioxidant and antinociceptive effects.

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