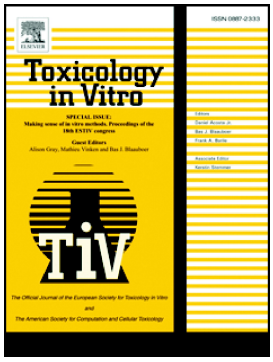


# Association of electroanalytical and spectrophotometric methods to evaluate the antioxidant activity of isobenzofuranone in primary cultures of hippocampal neurons

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**Abstract**

The isobenzofuran-1(3*H*)-ones (phthalides) exhibit various biological activities, including antioxidant activity on reactive oxygen species (ROS). An excess of ROS that cannot be naturally contained by cellular enzymatic systems is called redox imbalance, which damage cell membranes, proteins, and DNA, thereby possibly triggering neuronal death in several neurodegenerative diseases. Considering our ongoing efforts to find useful compounds to control redox imbalance, herein we evaluated the antioxidant activity of two phtalides (compounds **3** and **4**), using primary cultures of hippocampal neurons. Spectrophotometric assays showed that compound **3** significantly reduced ($p \leq 0.05$) ROS levels and lipid peroxidation compared to the control treatment, while compound **4** was unable at any of the tested concentrations. Despite their structural similarity, these compounds behave differently in the intracellular environment, which was reliably corroborated by the determination of oxidation potentials via cyclic voltammetry. It was demonstrated that compound **3** presents a lower oxidation potential. The combination of the mentioned methods allowed us to find a strong correlation between the chemical structure of compounds and their biological effects. Taking together, the results indicate that compound **3** presents desirable characteristics to act as a candidate pharmacological agent for use in the prevention and treatment of neurodegenerative diseases.

**Keywords:** spectrophotometric methods, cyclic voltammetry, isobenzofuranones, hippocampal neurons, free radicals, antioxidants.

## 1. Introduction

Primary cell cultures, specifically those of hippocampal neurons, obtained from a region associated with higher cognitive functions, such as learning and memory, are quite relevant for studies regarding pathogenic mechanisms involved in neurodegenerative diseases. Unlike cell lines of the nervous system, in primary cultures, hippocampal neurons become polarized, with developed axons and dendrites, facilitating the formation of functional synaptic connections [1,2]. The development of physiological characteristics similar to those observed *in vivo* results in these cultures being widely used for studies in the field of neurobiology, including as a model for studying the neurotoxicity of free radicals as well as the interaction between reactive oxygen species (ROS) and protective effects of compounds with antioxidant properties [3,4]. Neurotoxicity triggered by redox imbalance is a common feature in the development of different types of neurodegenerative diseases. Therefore, compounds that can act as preventive treatments have extensively been tested primarily using spectrophotometric techniques [5,6,7].

Development of new drugs that are capable of curbing redox imbalance and the consequent damage to cell membranes, proteins, and DNA, without causing neuronal toxicity, remains a challenge in the field of neurobiology. Considering this context, organic synthesis has emerged as an alternative that allows structural modifications in compounds that can perform well in both *in vitro* and *in vivo* assays. For instance, in isobenzofuran-1(3*H*)-ones (phtalides) the introduction of functional groups at specific molecular sites lead to antiplatelet, cytotoxic, GABA receptor-antagonistic, antimicrobial, antifungal, or antioxidant activity [8], demonstrating that structural similarities between compounds do not necessarily indicate effectiveness or similar function.

Electroanalytical techniques, such as cyclic voltammetry (CV), can assist in the selection of compounds with neuroprotective activity on redox imbalance. CV analyzes redox potential based on the linear scanning of a wide range of potentials over a time period [9]. Combined with the fact that most antioxidants present high electroactivity, CV facilitates a simple method for the selection of organic compounds with greater antioxidant capacity and to understand differences in antioxidant activity between structurally similar compounds in spectrophotometric tests. In addition, CV is a relatively simple instrumentation technique that can generate quick and highly reliable results at a low cost and using a small sample volume.

Considering of our ongoing efforts to find compounds to be used in the control of the redox imbalance, and therefore potential pharmacological agents for use in the prevention and treatment of neurodegenerative diseases, we herein describe the evaluation of the antioxidant performance on primary cultures of hippocampal neurons of chemically similar 3-(2-hydroxy-6-oxocyclohex-1-en-1yl)isobenzofuran-1(3*H*)one and 3-(2-hydroxy-4-methyl-6-oxocyclohex-1-en-1yl)isobenzofuran-1(3*H*)one, which are henceforth referred to as compounds **3** and **4**, respectively. The difference between these compounds is the methyl group, which is present in the structure of **4** and absent in **3**. It is demonstrated that the combination of spectrophotometric assays and CV analyses allowed us to establish a strong correlation between the chemical structure of the compounds and their biological effects. In addition to the analysis of these compounds, the 3-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1yl)isobenzofuran-1(3*H*)one, designated as compound **1**, which has previously been characterized by Ribeiro et al. [10] as having good antioxidant performance on primary cultures of hippocampal neurons using spectrophotometric techniques, was evaluated with CV in the present study and used as a positive standard of antioxidant activity. Thus, the aim of the present study was to associate electroanalytical and spectrophotometric methods to evaluate the antioxidant activity of

compounds **3** and **4** and to determine their potential as candidate pharmacological agents for use in the prevention and treatment of neurodegenerative diseases.

## 2. Material and methods

### *2.1. Samples and reagents*

For the cell culture, *L*-glutamine, minimal essential medium, neurobasal medium, glucose, Hank's saline solution, and B-27 supplement were purchased from Gibco Life Technologies (New York, NY, USA). BODIPY-C11, 2.7-dichlorofluorescein diacetate, sodium pyruvate, penicillin and streptomycin solution, 30% hydrogen peroxide ($H_2O_2$), poly-*L*-lysine, DNase solution, and trypsin (0.25%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine fetal serum was purchased from Cultilab (Campinas, Brazil). The MTT dye—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide—was purchased from Invitrogen (Eugene, OR, USA). Dimethylsulfoxide (DMSO) was purchased from LGC Biotecnologia (Cotia, Brazil). Hydrochloric acid (HCl) was purchased from Imprex (Varginha, Brazil). Ethyl alcohol was purchased from Vetec (Rio de Janeiro, Brazil).

### *2.2. Instrumentation*

Absorbance readings and fluorescence measurements were performed in a microplate reader (Multidetector VICTOR™ X3, Perkin-Elmer). Fluorescence images were taken with Zeiss LSM780 confocal microscope. For the electrochemical measurements, a potentiostat/galvanostat model PGSTAT 128N (Metrohm-Autolab) was used, controlled by NOVA software version 1.10.

### *2.3. Preparation and structures of isobenzofuranones*

The isobenzofuranones 3-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)isobenzofuran-1(3*H*)-one (**1**), 3-(2-hydroxy-6-oxocyclohex-1-en-1-yl)isobenzofuran-1(3*H*)-

one (**2**), and 3-(2-hydroxy-4-methyl-6-oxocyclohex-1-en-1-yl)isobenzofuran-1(3*H*)-one (**3**) herein investigated were prepared as previously reported [8]. Shortly, the compounds were obtained via condensation reactions, in the presence of DBU, between 1,3-diketones and phtalaldehydic acid.

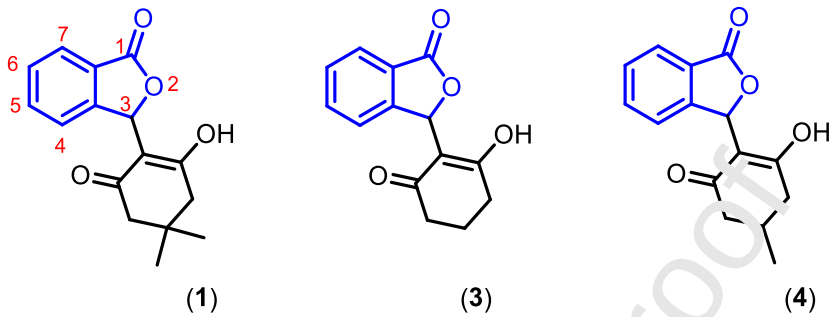


Fig. 1. Chemical structure of isobenzofuranones. The lactone ring fused to the benzene ring is highlighted in blue. Compounds **3** and **4** were synthesized for this study. Compound **1**, the positive antioxidant parameter, was previously tested by Ribeiro et al. (2020) using spectrophotometric techniques only.

*2.4. Cell culture*

Male and female C57BL/6 mice were maintained in the Animal Science Center of the Federal University of Ouro Preto (Ouro Preto, Minas Gerais State, Brazil). The animal care and use committee of Federal University of Ouro Preto approved the protocol (9659240418). Primary cultures of hippocampal neurons from embryonic day 17 (E17) C57BL/6 mouse embryos were obtained as previously described [11]. Briefly, the hippocampi were dissected in cold Hanks' balanced salt solution. The hippocampi were first incubated with trypsin (0.25%) for 20 minutes at 37 °C. After that, the trypsin was neutralized with plating medium (Eagle's minimum essential medium (10%), FBS and antibiotics (100 mg/mL streptomycin and 100 units/mL penicillin). Afterwards, the hippocampi were dissociated mechanically in plating medium. The cells were then plated at a density of 5.0 x $10^4$ or 1.0 x $10^5$ cells/well on dishes pre-coated with 10 mg/mL poly-*L*-lysine and maintained at 37 °C and 5% $CO_2$. The plating medium was replaced by maintenance medium (Neurobasal medium, B-27, L-glutamine, 100 mg/mL streptomycin and 100 units/mL penicillin) 4 h after the cells were plated. The experiments were

carried out 6 to 10 days after the neurons were seeded onto the dishes at an appropriate density according to each experimental scale.

*2.5. Isobenzofuranone treatment of the primary hippocampal neurons*

For each evaluated compound in this work (compounds **3** and **4**, Fig. 1), a 2 mM stock solution in DMSO (20% v/v) was prepared. The stock solution was diluted to obtain concentrations of 50, 100 and 150 µM of the compounds. To control for the cytotoxicity of DMSO, its final concentration was kept at 0.05% v/v for all treatments. The neurons were seeded onto 96-well dishes. After 6 days, the cells were treated with 50, 100 and 150 µM of isobenzofuranones and incubated for 2 h in a humidified chamber at 37 °C with 5% $CO_2$. After the treatment with compound **3** and **4**, redox imbalance was induced for 3 h with hydrogen peroxide (100 µM) [12,13].

*2.6. Measurement of cell metabolic activity*

The metabolic activity of hippocampal neurons was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [10]. Briefly, the neurons were pre-treated for 2 h with compounds **3** and **4** at concentrations of 50, 100 and 150 µM in 96-well dishes ($5.0 \times 10^4$ cells/well). After that, redox imbalance was induced with 100 µM of hydrogen peroxide for 3 h. Then, the medium was replaced with 2.5 mg/mL MTT, and the dish was incubated for 4 h at 37 °C. Subsequently, the dish was incubated with 10% SDS solution for 16 h at 37 °C to solubilize the formazan salt. Finally, the absorbance was measured at 530 nm with a microplate reader.

*2.7. Cell viability*

For the cell viability, the LIVE/DEAD® (Thermo Scientific) assay was used according to manufacturer's instructions. Briefly, mouse primary hippocampal neurons were seeded onto poly-L-lysine-coated 4-well dishes at density of $1.0 \times 10^5$ cells/well and cultured for 6 days. On day 6, neurons were incubated for 2 h with compounds **3** and **4** at concentrations of 50, 100 and

150 µM. Afterwards, two reagents, calcein-AM and ethidium bromide, were added to cultures in their usual media. As an indicator of cell viability, the calcein-AM is metabolically converted by intracellular esterase activity resulting in the green fluorescent product, calcein. Ethidium bromide is excluded from live cells but is readily taken up by dead cells and stains the DNA. Live and dead cells were counted from fluorescence images taken with Zeiss LSM780 confocal microscope at 20X magnification. The cell viability rate was calculated as the percentage of viable cells in the total amount of cells.

*2.8. Measurement of intracellular ROS accumulation*

Intracellular ROS production was assessed by a fluorescence assay that employed 2,7-dichlorofluorescein diacetate ($H_2$DCFDA) as previously described [10]. Briefly, after pre-treatment with compounds **3** and **4** for 2 h and the induction of redox imbalance, the medium was replaced by an $H_2$DCFDA (2 µM) solution for 45 minutes. After that, the wells were washed three times with colourless calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) free Hanks' balanced salt solution. Fluorescence was monitored every 30 minutes for 3 h after the addition of 100 µM $H_2O_2$. Fluorescence values were measured using a microplate reader at 485/535 nm excitation/emission wavelengths.

*2.9. Measurement of Lipid Peroxidation*

To analyse the membrane damage caused by redox imbalance induction, we used the protocol as previously reported [10]. Mouse primary hippocampal neurons were seeded at a density of 5.0 x $10^4$ cells/well and cultured for 6 days. On day 7, the cells were pre-treated with compounds **3** and **4** for 2 h. After that, the medium was replaced by a solution containing BODIPY$_{581/591}$ C11 (2 µM) and incubated for 30 min. Then, the wells were washed three times with colourless $Ca^{2+}$ and $Mg^{2+}$ free Hanks' balanced salt solution. Before reading the results, redox imbalance was induced by the addition of 100 µM $H_2O_2$. Readings were performed every

30 minutes for 3 h, with the first reading at time 0'. Fluorescence values were measured using a microplate reader at 485/535 nm excitation/emission wavelengths.

*2.10. Cyclic voltammetry*

*2.10.1. Sample preparation*

For the CV analyses, compounds **3** and **4** were prepared and diluted to a $1 \times 10^4$ µM final solution. The samples of the compounds were homogenized in 500 µL of DMSO solvent. The same procedure was followed for compound **1** (Fig. 1), which was used as a standard for the analysis of oxidation potentials. Thereafter, a 50 µL sample of each of compounds **1**, **3**, and **4** was deposited on glass substrates coated with a conductive layer of indium tin oxide (ITO) with a specific resistance of 4–8 Ω/square. For film formation, the sample solutions were deaerated using an inert gas ($N_2$) for 10 min. The area occupied by the film formed was calculated by measuring the surface previously delimited using a tape (Masterfix).

*2.10.2. Electrochemical assays*

Before starting measurements, the electrodes were thoroughly washed with Milli-Q water and immersed in 0.1 M HCl solution in an electrochemical cell at a temperature of 25.0 ± 0.5 °C. All measurements were carried out using the conventional three-electrode system, composed of a working electrode, the sample, an auxiliary platinum electrode, and a saturated calomel reference electrode. CV scans were obtained from −1.8 V to 0.0 V, with a scanning speed of 0.1 V/s. All voltamograms were performed six times [14].

*2.11. Statistical analysis*

A minimum of three independent replicates were performed, using three different primary cultures of hippocampal neurons obtained from three different experiments for each experimental condition, and the results presented were representative of these replicates (n = 9).

The results are presented as mean ± standard deviation (SD). Two-way ANOVA analyses were performed, followed by Tukey's post-test for parametric data. For non-parametric data, the Kruskal–Wallis test, followed by Dunn's post-test, was used for analysis. The difference between the data was considered statistically significant at a p value of $\leq 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 software.

## 3. Results and discussion

### *3.1. Cell vitality*

Isobenzofuranones, particularly those functionalized at the C3 position of the $\gamma$-lactone ring, are a class of compounds known for their diverse biological activities [15], such as anti-HIV [16], antiallergic [17], antifungal [18], antidiabetic [19], insecticidal [20,21], anti-inflammatory [22], antispasmodic [23], COX-2–inhibitory [24], antimicrobial [25], herbicidal [26], antioxidant and antiplatelet [27], leishmanicidal [8], and anticancer [28].

Our research group has been investigating structural aspects [29–33] and bioactivity of isobenzofuranones [8, 34–37]. Recently, we demonstrated that the isobenzofuranone that we termed as compound **1** (Fig. 1) exhibited significant effects on the $H_2O_2$-induced redox imbalance in hippocampal neurons [10]. With an aim to continue our investigations on the effect of isobenzofuranones on redox imbalance, we decided to evaluate the effects of compounds **3** and **4** (Fig. 1); these compounds present similar structural formulas but with different substituents and may thus result in differences in the type of biological activity exhibited by them.

Compounds **3** and **4** were initially evaluated for their toxicity on hippocampal neuron cultures. To this end, the cell vitality test was performed using the MTT assay and the results showed different behaviors for both compounds. Compound **3** showed no significant difference

between the control group and different concentrations, indicating that it did not reduce neuronal metabolism. However, despite the structural similarity to compound **3**, compound **4** caused a significant reduction in neuronal metabolism at concentrations of 100 and 150 µM (Fig. 2).

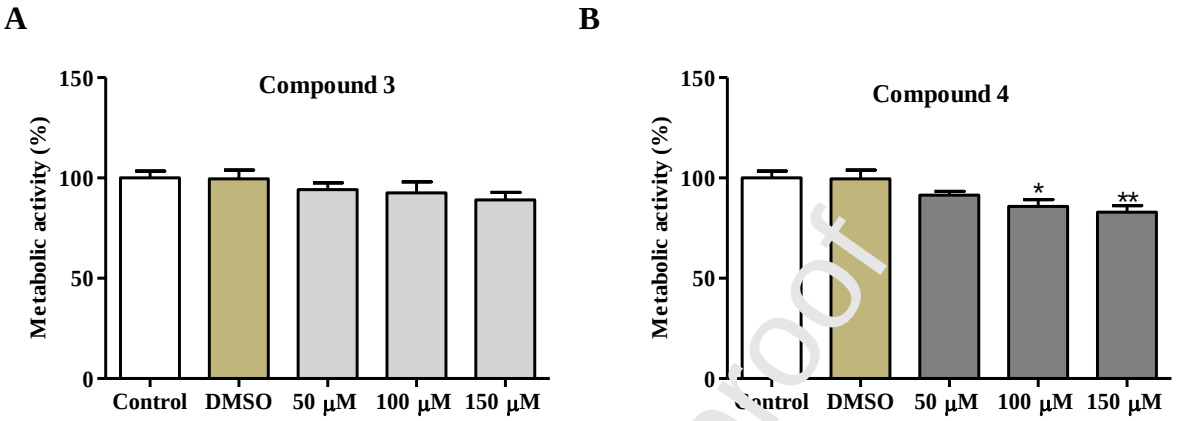


Fig. 2. Metabolic activity measurement of hippocampal neurons. Graphs show the percentage of cell vitality after treatment with compounds **3** and **4**. Data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by the Tukey post-hoc test. *p ≤ 0.05 and **p ≤ 0.01 compared with the control.

The live/dead assay, which measures intracellular esterase activity and plasma membrane integrity, was employed to assess the survival of neurons treated with the compounds **3** and **4**. The morphology and the number of live cells observed in the presence of the compounds **3** and **4** does not differ from the control groups (Fig. 3A and 3B). As shown in Fig. 4, cell viability exhibited no significant differences between treated and control groups ($p < 0.05$) for all tested concentrations of the compounds **3** and **4** after 2 h of treatment. The live/dead results showed that although the compound **4** reduces cell metabolism, it does not interfere with neuronal viability.

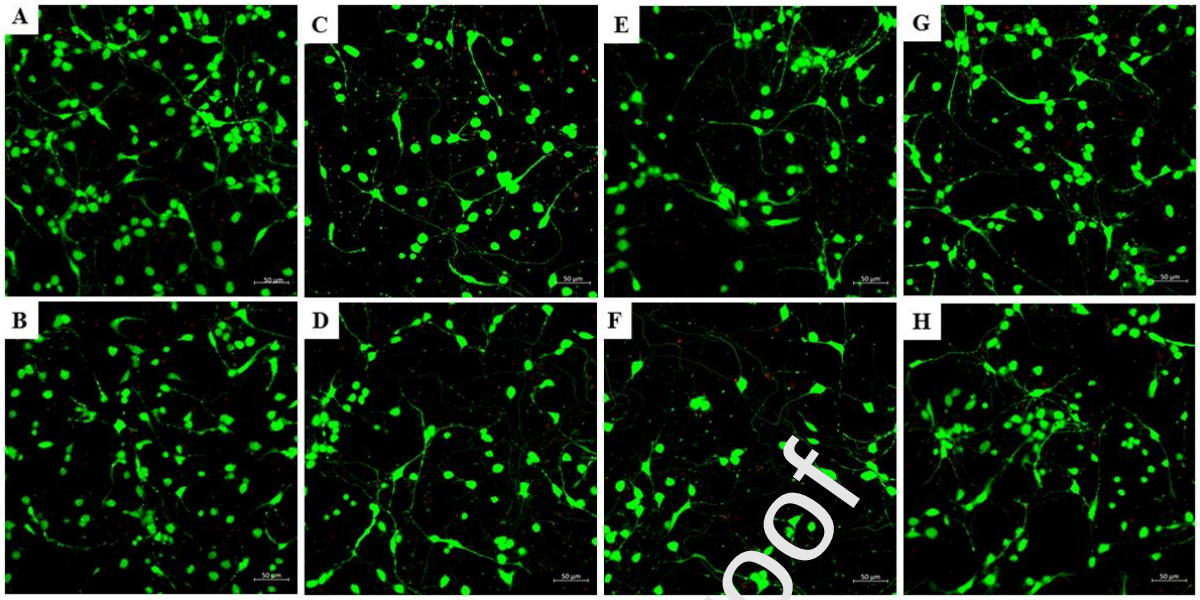


Fig. 3. The images shown in A-H were taken of live cells at 20x magnification. A comparison of control (A), DMSO control (B) and hippocampal neurons treated with compounds **3** (C, E and G) and **4** (D, F and H). Live cells were measured by the enzymatic conversion of permeant calcein-AM to fluorescent calcein (green). Dead cells were detected by the uptake of ethidium bromide into cell DNA (red). Bar, 50 μm.

**A** **B**

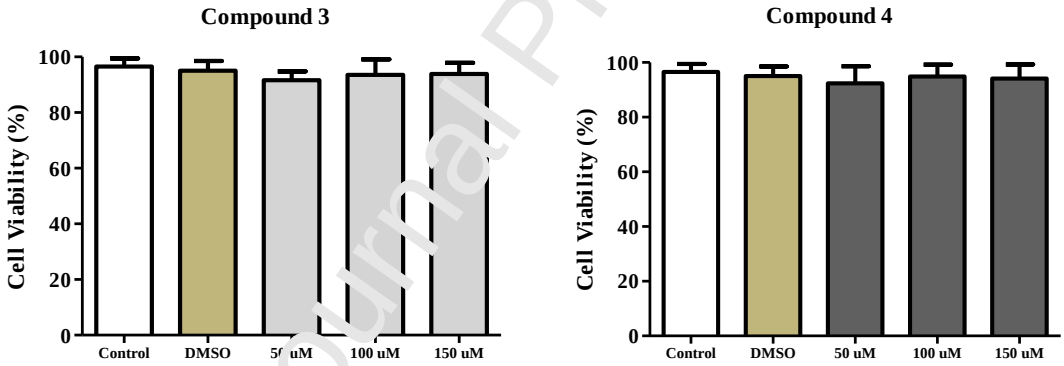


Fig. 4. Cell viability assay of hippocampal neurons. Graphs show the percentage of cell viability after treatment with compounds **3** and **4**. Data represent the mean ± SD of two independent experiments conducted in triplicate. One-way ANOVA followed by the Tukey post-hoc test.

Further, compounds **3** and **4** were evaluated in relation to pretreatment subjecting them to an induced redox imbalance. The results revealed that the compounds were unable to improve the neuronal cell vitality, which was reduced after the cellular injury caused by $H_2O_2$ (Fig. 5).

**A** **B**

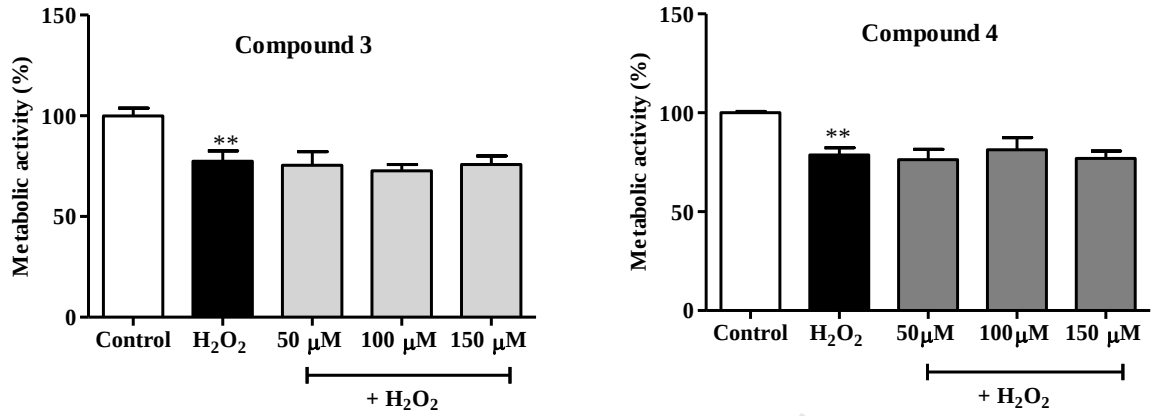


Fig. 5. Metabolic activity measurement performed using MTT assay of hippocampal neurons submitted to redox imbalance. Graphs show the percentage of living cells after 2 h of pretreatment with compounds **3** and **4**. The data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by the Tukey post-hoc test. **$p \leq 0.01$ compared with the control.

### *3.2. Intracellular ROS accumulation*

Intracellular ROS increase and accumulation triggers neuronal cell death in several neurodegenerative diseases [38]. In Alzheimer's disease, for instance, redox imbalance primarily affects the hippocampus, a region involved in the formation of short- and long-term memory and in spatial navigation, in addition to being extremely important to learning [39,40]. Considering this context, compounds **3** and **4** were evaluated with respect to the possibility of inducing ROS formation. This possibility was ruled out according to the results of the experiments measuring ROS production (Fig. 6)

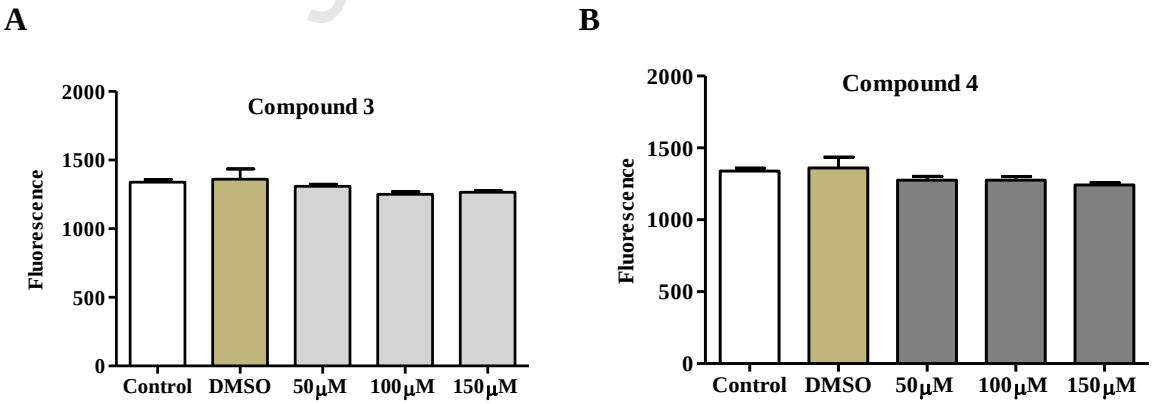


Fig. 6. Measurement of ROS. Graphs show ROS levels produced in hippocampal neuron cultures after treatment with compounds **3** and **4**. Data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by Dunn's multiple-comparison post-hoc test. *$p \leq 0.05$.

Because compounds **3** and **4** did not induce ROS formation (Fig.6), they were tested as a pretreatment for neurons subjected to redox imbalance (Fig.7). The results of these tests showed that compound **3** at a concentration of 150 µM led to a significant reduction in ROS levels compared with the control sample treated only with $H_2O_2$ (Fig. 7A). Compound **4** was unable to reduce ROS levels at any of the tested concentrations (Fig. 7B). These results indicate that despite their structural similarity, these compounds behave differently in the intracellular environment. As mentioned earlier, compound **3** did not exhibit toxicity to the hippocampal neurons (Fig. 2).

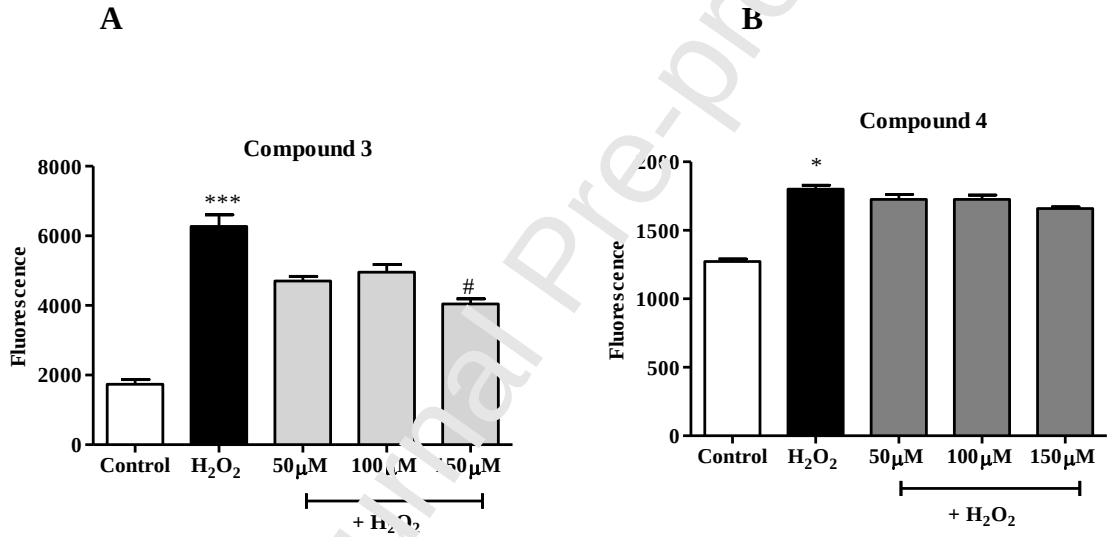


Fig. 7. Measurement of ROS after induction of redox imbalance. Graphs show ROS levels produced in hippocampal neuron cultures after pretreatment for 2 h with compounds **3** and **4**. Data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by Dunn's multiple-comparison post-hoc test. *$p \leq 0.05$ and ***$p \leq 0.001$ compared with the control. $^{\#}$ $p \leq 0.05$ compared with the $H_2O_2$ group.

*3.3. Lipid peroxidation*

Neurons contain large amounts of polyunsaturated fatty acids in their membranes. These fatty acids are peroxidation targets when exposed to excess ROS produced by redox imbalance [41–43]. Compounds **3** and **4** did not promote lipid peroxidation (Fig. 8).

**A** **B**

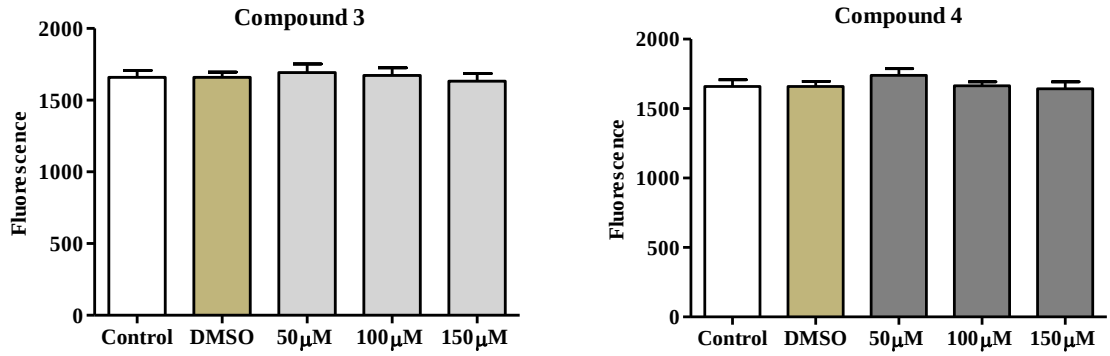


Fig. 8. Measurement of lipid peroxidation. Graphs show lipid peroxidation levels in hippocampal neuron cultures after treatment with compounds **3** and **4**. Data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by Dunn's multiple-comparison post hoc test. *$p \leq 0.05$

Therefore, they were tested as a pretreatment for neurons before the induction of redox imbalance (Fig. 9). The results obtained corroborate the findings of Gao et al. [42] and Xu et al. [44] for the substances 3-butyl-6-bromo-1(3*H*)-isobenzofuranone and 3-butyl-6-fluoro-1(3*H*)-isobenzofuranone, respectively, and shows that compound **3**, at a concentration of 150 µM, caused a significant reduction in lipid peroxidation compared with the control sample treated only with $H_2O_2$ (Fig. 9A). This protection against membrane damage is possibly correlated with the ability of this compound to reduce ROS levels, which is a desirable characteristic when screening compounds as candidate pharmacological agents for use in the prevention and treatment of neurodegenerative diseases [45]. Contrary to compound **3**, pretreatment with compound **4** showed no efficacy in limiting lipid peroxidation in any of the three concentrations tested (Fig. 9B).

The results of the spectrophotometric analyses differed with regard to the activity of compounds **3** and **4** on the primary cultures of hippocampal neurons. To further elucidate these results, electroanalytical tests were performed.

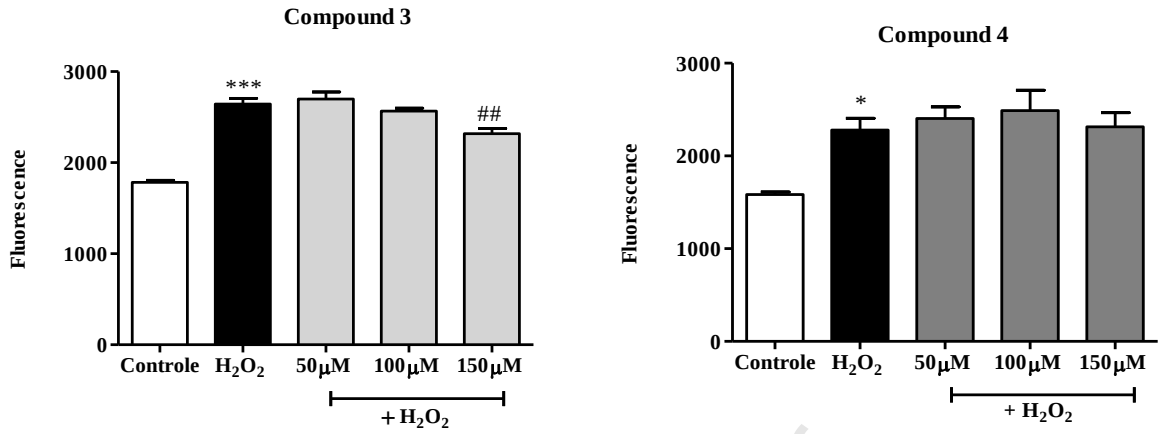


Fig. 9. Measurement of lipid peroxidation after induction of redox imbalance. Graphs show lipid peroxidation levels in hippocampal neuron cultures after 2 h of pretreatment with compounds **3** and **4**. Data represent the mean ± SD of three independent experiments conducted in triplicate. One-way ANOVA followed by Dunn's multiple-comparison post-hoc test. *$p \leq 0.05$ and ***$p \leq 0.001$ compared with control. #$p \leq 0.01$ compared with the $H_2O_2$ group.

### *3.4. Cyclic voltammetry*

Cyclic voltammetry was used for a comparative analysis of the antioxidant activity of the compounds. The redox potential of compounds **3** and **4** was evaluated using a third isobenzofuranone—called compound **1** (Fig. 1)—as a positive pattern. Compound **1** was previously evaluated using spectrophotometric methods and presented significant antioxidant activity in primary cultures of hippocampal neurons [10]. Compounds **1**, **3**, and **4** have chemical and structural similarities that classify them as phthalide derivatives due to the presence of an aromatic ring bound to the lactone portion ($C_8H_6O_2$).

Cyclic voltammograms were obtained as results of the redox processes involving the species undergoing oxidation—the anodic pathway—and species undergoing reduction almost reversibly—the cathodic pathway. The analytical peaks obtained as a response from the cyclic system were attributed to an oxidative depletion of antioxidant molecules in the electrode. Thus, the oxidation potentials (Ap) facilitated a comparative analysis of the antioxidant activity of the different compounds, i.e., the ability of the compound to be oxidized and thus protect the cells from the harmful effects of oxidative stress [46].

The voltammogram for the standard compound **1** (Fig. 10) was obtained from readings in the scan range from −1.8 to 0 V, at a scanning speed of 0.1 V/s. The analysis revealed a negative anodic peak potential or oxidation potential of −0.43 V for compound **1**. Despite expanding the scanning range, no other peak was detected, proving the existence of only one oxidation peak. The voltammograms of compounds **3** and **4** (Fig. 10), obtained under the same conditions as those of compound **1**, presented oxidation potentials of −0.41 V and −0.36 V, respectively. A high negative value of oxidation potentials reflects a greater ability of a given molecule to donate electrons and accordingly demonstrates its significant antioxidant activity [47]. Therefore, the voltammograms showed that compound **1** had a lower potential and thus presented the highest antioxidant activity among the evaluated compounds, which was the expected result. Compound **3** presented an oxidation potential similar to that of compound **1**. Compound **4** presented a higher oxidation potential compared with other compounds, which reflects its lower antioxidant activity. These results corroborate the spectrophotometric assays performed *in vitro* and the results presented by Ribeiro [10] for compound **1**.

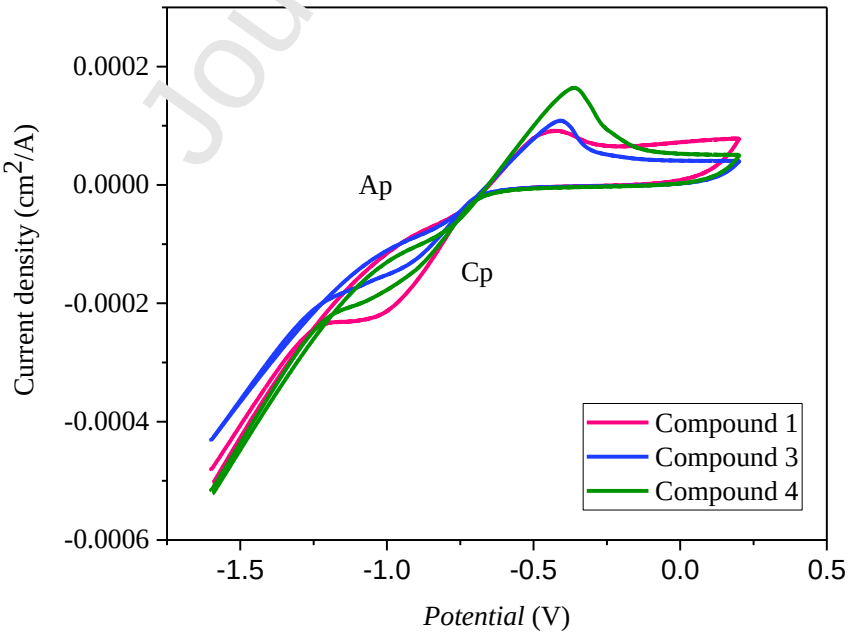

Fig. 10. Cyclic voltammogram of compounds **1**, **3** and **4** (1 x $10^4$ μM) obtained using a working electrode. The compounds **1**, **3** and **4** were dissolved in DMSO. Ap (anodic process) and Cp (catodic process). Scan rate: 0.1 V $s^{-1}$.

The isobenzofuranones are prone to give rise to stable isobenzofuranyl radicals. The stability of these radicals is related to electronic delocalization of the unpaired electron as depicted in Fig. 11.

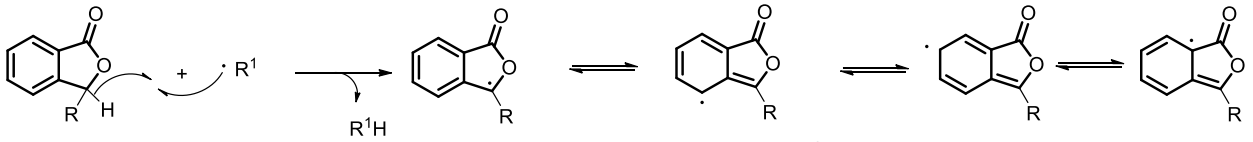


Fig. 11. Formation of isobenzofuranyl radical and electronic delocalization of it.

This property of isobenzofuranones is associated with their antioxidant activity and they have been explored as stabilizers to prevent thermal and oxidative decomposition during the processing of polymers at high temperatures [48,49]. Other reports have described the antioxidant activity of isobenzofuranones [27,50]. Within this context, it is plausible to assume that isobenzofuranones react with hydroxyl radicals (·OH) (Fig. 12), which is the main species involved in the induced redox imbalance in the hippocampal neuron model. The hydroxyl radicals, generated from $H_2O_2$, are very toxic species to the cells. Therefore, the isobenzofurenones can capture this species and decrease the concentration of them in the intracellular media [51]. The generated isobenzofuranonyl radicals can dimerize or react with another radical [48].

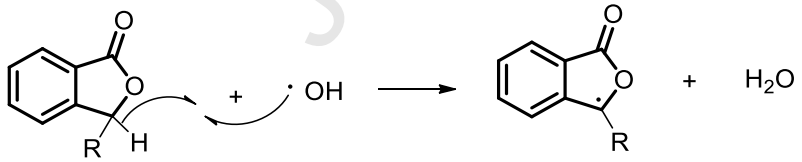


Fig. 12. Reaction between isobenzofuranone and hydroxyl radical with formation of isobenzofuranyl radicals.

One important point is that the stability of the isobenzofuranonyl radicals depend on the groups attached do benzylic carbon (in Fig. 9 and 10, a group attached to the benzylic carbon is designed as R) [52]. This fact is in agreement with the findings described in the present investigation in which we observe different biological responses of isobenzofuranones presenting different alyciclic groups attached to the benzylic carbon of the isobenzofuranone nucleus.

## 4. Conclusion

In summary, using conventional spectrophotometric and electroanalytical methods, we investigated the antioxidant properties of isobenzofuranones presenting different alicyclic groups in their structures. The spectrophotometric method facilitated the evaluation of isobenzofuranones with respect to their effects on redox imbalance induced in hippocampal neurons. By determining oxidation potentials via CV, it was possible to establish a strong correlation between the chemical structure of the compounds and the biological effects observed. Therefore, the combination of methods used in this investigation is useful in the selection of isobenzofuranones aiming at their application as antioxidants.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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