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Cytoprotective Effect of *Valeriana officinalis* Extract on an In Vitro Experimental Model of Parkinson Disease

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Abstract Parkinson's disease (PD) is one of the most important neurodegenerative worldwide disorders. The potential cytoprotective effects of aqueous extract of *Valeriana officinalis* on rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells were demonstrated. The cytotoxicity, cell viability and analysis of cellular morphology were performed by MTT-tetrazole (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and phase contrast microscopy, respectively. Significant changes in the cellular morphology, and condensation of the cell body could be observed when cells were treated with 300 nM rotenone for 48 h. Three different concentrations of *Valeriana officinalis* extract were used (0.049, 0.098 and 0.195 mg/mL). These extracts brought about an increase of $7.0 \pm 1.3\%$, $14.5 \pm 1.3\%$ and $14.5 \pm 3.2\%$ in cell viability. Our results indicated that neuroprotector action of the *Valeriana officinalis* extract provides support for later studies as they help understanding this drug for the development of cytoprotective various therapies in PD.

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Introduction

Parkinson's disease is a complex chronic neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the substantia nigra [1] and marked by formation of ubiquitin- and α-synuclein-positive cytoplasmic inclusions known as Lewy bodies [2]. Clinical symptoms include motor abnormalicies (tremor, rigidity, slowness, balance problems), autonomic disturbances, psychiatric sequelae (usually depression), and cognitive impairment [3]. It is one of the most common neurodegenerative disorders, only second to Alzhemier's disease [4], affecting 1–2% of the population older than 65 [5]. The cause of this disorder remains unknown. However, it appears that most cases of PD will have multifactorial etiologies, with both genetic and environmental components [6, 7]. Several risk factors have also been described. An important element previously described is the significant association of occupational exposure to herbicides and insecticides with Parkinson's disease [8].

Despite different possible causes of PD, the pathogenesis of this disease appears to be converging on common mechanisms: mitochondrial impairment, oxidative stress and protein mishandling [3]. Since oxidative processes are an important factor in the pathogenesis of several disorders, including neurodegenerative diseases [9], one model to study PD is the administration of the plant-derived pesticide rotenone which is a specific inhibitor of mitochondrial complex I, inducing apoptosis by generating mitochondrial reactive oxygen species, in PC12, HT1080, HEK-293 and SH-SY5Y cell lines and originating effects in rats which closely resemble PD [10–14].

Therapeutic efforts aimed at removal of free radicals or prevention of their formation may be beneficial in PD. Regarding this point, natural products are an attractive source of chemical structures enjoying potent biological activities with promising pharmacological profiles.

The crude extract of *Valeriana officinalis* is widely used in many countries: There are at least 25 products containing it in the United Kingdom and over 400 in Germany [15]. The genus *Valeriana*, with about 200 species, belongs to the Valerianaceae family and grows throughout the world [16]. In Brazil, *Valeriana officinalis* has been used in traditional medicine for its sedative, anticonvulsant, hypnotic effects, and anxiolytic activity [17]. This drug has also been used to treat digestive problems and urinary tract problems for at least 1,000 years [18].

Several reports have suggested that flavanoids and other compounds of natural origin could be useful to protect cells from rotenone toxicity [13, 19–21]. *Valeriana officinalis* contains over 150–200 chemical constituents [22], including flavonoids with activity on the CNS [23] and lignans with antioxidative and vasorelaxant activities [16]. Moreover, neuroprotective properties of *Valeriana officinalis* extracts in rat hippocampal neurons have been described [24]. Thus, considering these data and the already known effects on CNS, we propose that *Valeriana officinalis* must be a target in the search for new agents assisting treatment of PD. The objective of this work is to offer evidences of the neuroprotective action of the aqueous extract of *Valeriana officinalis* in an in vitro experimental model of PD to provide support to later studies with this drug.

Experimental Procedures

Materials

Neurobasal medium DMEM HAM F-12, fetal bovine serum, and penicillin/streptomycin antibiotics were obtained from Cultilab (São Paulo, Brazil). MTT-tetrazole (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), sodium dodecylsulfate (SDS), dimethylsulfoxide (DMSO) and bovine serum albumin were from Sigma–Aldrich (St. Louis, MO, USA). Human neuroblastoma SH-SY5Y cells were kindly provided by Dr. Pablo Mendez, Instituto Cajal, Madrid, Spain.

Valeriana officinalis came from Laboratório de Farmacognosia I (Faculdade de Farmácia, Universidade Federal da Bahia, Brazil) as a powder from pulverized rhizome of the plant. To prepare the extract used in the experiments, 250 mg of this powder were diluted in 2 ml water, heated to 100°C for 10 min. in the water bath and centrifuged at 672g for 10 min. Supernatants were filtered through membrane FG hydrophilic with 0.2 μM pore (MILLI-PORE, Billerica, MA, USA).

Cell Culture

Human neuroblastoma SH-SY5Y cells were cultured at 37°C in a DMEM HAM F-12 medium supplemented with 10% fetal bovine serum, 100 UI/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 2 days. Cells were seeded in 96-well plates at a density of 62.5×10^3 cell/cm^2 and were cultured for 72 h.

Rotenone-induced Oxidative Damage in SH-SY5Y Cells

First of all, we tested the best experimental conditions. In a pilot investigation cells were treated with rotenone dissolved in DMSO at concentrations ranging from 10 nM to 1,000 nM for 24, 48, 72 h and cell viability examined. Treatment of SH-SY5Y cells with rotenone caused cell death in a concentration- and time-dependent manner [29]. In this article cells were treated following the procedure described by Valverde et al. [29]: SH-SY5Y cells were incubated with 300 nM rotenone dissolved in DMSO and mixed with the culture medium (final DMSO concentration of 0.5% v/v) in the presence or absence of *Valeriana officinalis* estract for 48 h.

Treatment with *Valeriana officinalis*

The extract of *Valeriana officinalis* was submitted to a serial dilution. To check possible toxic effects, SH-SY5Y cells were treated with the extract in concentrations ranging from 0.049 mg/ml to 6.250 mg/ml (final concentration after dilution with the medium). Cell viability was examined by MTT assay. Data obtained from this experiment were fitted to a non-linear regression plot to determine the IC_{50} for the extract at 48 h. The MTC (minimal toxic concentration, minimal concentration, among those used, which allows checking whether there is a significant difference between the treated and the control group) was also determined. After that, four concentrations (non-toxic) were chosen for the evaluation of the possible neuroprotective effect. This evaluation was performed by means of a treatment with both rotenone at 300 nM and aqueous extract of *Valeriana officinalis* at different concentrations.

Analysis of Cell Viability

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

The MTT is a yellow substratum for mitochondrial dehydrogenases in a surviving cell that generates a blue product (formazan) measurable by photometric techniques [25].

Human neuroblastomas SH-SY5Y cells in a 96-well plate were submitted to the treatments selected and after this, the MTT reagent dissolved in PBS was added to each well (0.04 mg/ml/well). Following an additional 2 h incubation period, 100 μl of SDS (50% DMF and 20% SDS in water) was added to break up the cells and to dissolve the formazan crystal, absorbance was then measured at a 595 nm of wavelength with a microplate reader (THERMO PLATE, model TP-reader—type B). Wells without cells were used as blanks and were subtracted from each sample as background. Results were expressed as a percentage of control and displayed in a graph.

Analysis of Cellular Morphology for Phase Contrast Microscopy

Cells in culture were observed with an inverted phase contrast microscope (Eclipse TS100, NIKON, Tokyo, Japan) and photographed with a digital camera (Coolpix4300, 4.0 megapixels resolution, NIKON, Japan) after the treatments to analyze morphological changes. The medium was changed before the observation to remove the dead cells that had lost adherence to the plate. A 10× objective was used.

Statistical Analysis

Data presented normal distribution. Thus, results are expressed as mean values (±SEM). The statistical evaluation of the data was performed by One-way analysis of variance (ANOVA) by means of a Dunnett Multiple Comparisons Test. A probability for all experimental values *$P < 0.05$, **$P < 0.01$ was accepted as an indication of statistically significant difference.

Results

Effects of *Valeriana officinalis* Extract on Cell Viability

Human neuroblastoma SH-SY5Y cells were treated with *Valeriana officinalis* extract in concentrations ranging from 0.049 mg/ml to 6.250 mg/ml to search for possible toxic effects (measured by MTT assay). The IC_{50} for aqueous extract of *Valeriana officinalis* was 2.805 mg/ml calculated from a dose-response curve (Fig. 1a) (the mathematic model is $V = -1235 + \{1340.8/[1 + 10^{(0.9153 \log C - 4.5188)}]\}$, in which V is percentage of cell viability and C is the extract concentration at μg/ml. The R^2 value was 0.9984. Treatment with the extract at concentrations up to 0.391 mg/ml had no
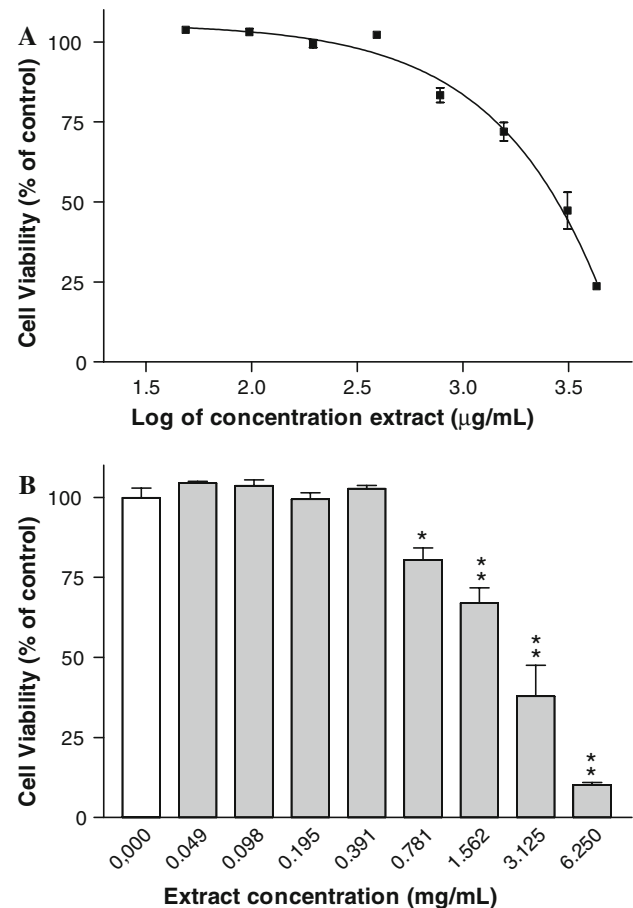

Fig. 1 Effects of aqueous extract of *Valeriana officinalis* against human neuroblastoma SH-SY5Y cells. (**a**) Dose-response curve to extract (by non-linear regression); concentrations of extract are expressed as Log of μg/ml and values of cell viability are stated as percentages of the control group. Equation: $V = -1235 + \{1340.8/[1 + 10^{(0.9153 \log C - 4.5188)}]\}$, $R^2 = 0.9984$. The value calculated for IC_{50} was 2.805 mg/ml. (**b**) Measurement of toxic effect of each concentration after serial dilution. Concentrations are expressed as mg/ml and cell viability as a percentage of the control group (first column). Treatment with the extract at concentrations up to 0.391 mg/ml did not show significant statistical difference between the cell viability of the control group. The MTC was 0.781 mg/ml. Then the concentrations used in the later experiment were not be equal or highest than this value. For statistical evaluations one-way ANOVA analysis, followed by a Dunnett Multiple Comparisons Test were used. *$P < 0.05$; **$P < 0.01$

effect on the viability of human neuroblastomas SH-SY5Y cells (Fig. 1b).

Measurement of the Cytoprotective Effect of *Valeriana officinalis* by MTT Essay

In order to study the cytoprotective effects of *Valeriana officinalis*, treatments were as follows: cells with: (a) Not treated control group; (b) rotenone (300 nM) and (c) rotenone (300 nM) + *Valeriana officinalis* (at 0.049 mg/ml, 0.098 mg/ml, 0.195 and 0.391 mg/ml).
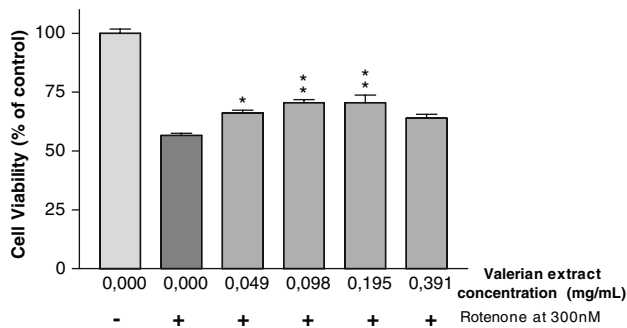
Fig. 2 Evidences of cytoprotective effects of aqueous extract of *Valeriana officinalis* on rotenone-induced cell damage by using MTT assay. The data are expressed as a percentage of the control. In all concentrations tested, except for the last one (0.391 mg/ml), as the extract of *Valeriana officinalis* brings about an increase in cell viability when compared with cells treated with rotenone alone. For statistical evaluations one-way ANOVA analysis, followed by a Dunnett Multiple Comparisons Test were used. $*P < 0.05$; $**P < 0.01$

In the first three tested concentrations, the extract causes an increase of $7.0 \pm 1.3\%$, $14.5 \pm 1.3\%$ and $14.5 \pm 3.2\%$ in the cell viability for each concentration, when compared with cells treated with rotenone alone (negative control). Then, it follows that *Valeriana officinalis* provides significant protection to SH-SY5Y cells against damage caused by rotenone (Fig. 2).

However, the extract at 0.391 mg/ml was not able to promote a statistically significant increase in cell viability.

Influence of Treatments on Cellular Morphology

Significant changes in cellular morphology were observed when cells were treated with 300 nM rotenone for 48 h. In this case, surviving cells showed retraction of cytoplasm and decrease in tack on the plate, in contrast with the typical morphology of cells from SH-SY5Y line presented by the control group.

This effect was partially reverted when the *Valeriana officinalis* extract was added to the treatment with rotenone. In these groups, the normal morphology of surviving SH-SY5Y cells being almost totally recouped (Fig. 3).
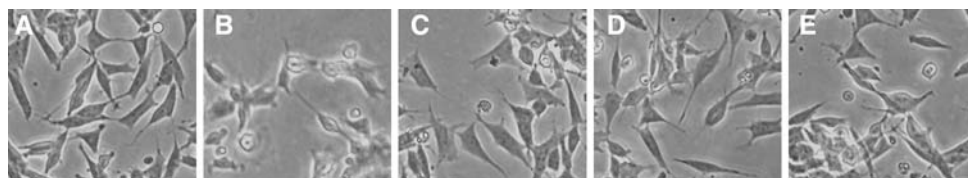
Discussion

Advances in the understanding of the neurodegenerative pathologies are originating new opportunities for the development of neuroprotective therapies. In this work, we have found that one aqueous extract of *Valeriana officinalis* causes significant cytoprotective effects on rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells.

Administration of the plant-derived pesticide rotenone is one of the most widely used model to study PD [10, 26–30]. Epidemiological studies have suggested a correlation between the exposure to pesticides and the increased risk of Parkinson's disease. Furthermore, toxicological data suggest that rotenone have neurotoxic actions which could play a role in the development of PD [14, 31–33]. The most significant pathological feature of PD is the presence of oxidative stress [34, 35]. The free radical generation may result directly from inhibition of the mitochondrial respiratory chain or indirectly during the apoptotic process itself. It has been suggested that rotenone-induced apoptosis in SH-SY5Y cells could be an in vitro model to study neurodegenerative diseases as PD [13, 36].

We have herein used neuroblastoma cells since they have many advantages, mainly as they are simpler and easier to culture than primary cultures of neurones, which may be too difficult, expensive, or unavailable. In those labs where their biochemistry has been studied extensively, these cells may be useful models for neurological disorders where no animal models of a disorder exist, furnishing clues on how such diseases occur [37].

The popularity of the widely used *Valeriana officinalis* extracts suggests that pharmacological effects of this plant must involve cellular and molecular targets in the central nervous system (CNS) [24]. However, current evidences are not enough to identify these targets [15].

In our experimental model, cell viability measured by the MTT method, showed that treatments with rotenone for 48 h reduce the survival of human neuroblastoma SH-SY5Y cells. Toxicity of the *Valeriana officinalis* extract was also analyzed and no toxicity was found at concentrations up to 0.391 mg/ml.

Fig. 3 Analysis of protective effect of aqueous extract of *Valeriana officinalis* by phase contrast microscopy. The pictures, obtained by phase contrast microscopy, show the effect of the treatment on cellular morphology. Cells treated with (**a**) vehicle (**b**) 300 nM rotenone, (**c**) rotenone and extract at 0.049 mg/ml, (**d**) 0.098 mg/ml and (**e**) 0.195 mg/ml (concentrations with which a significant protective effect was found). Changes in the cellular morphology were observed in b, which was partially reverted when the *Valeriana officinalis* extract was added to the rotenone treatment

The cell viability assays clearly show that the aqueous extract of *Valeriana officinalis* has the ability to protect SH-SY5Y cells against damage induced by rotenone. Among the four no-toxic concentrations tested, only the last (the highest) does not show this capacity, stopping the initial dose-response behavior of the effect. If the molecule (or molecules) responsible for *Valeriana officinalis* neuroprotection is isolated, a continuous dose-response behavior will probably be found.

The mechanism involved in the cytoprotective effects of *Valeriana officinalis* in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells remains to be delineated. However, there are several possibilities. In vitro, constituents of *Valeriana officinalis* mediate the release of γ-aminobutyric acid and bind the same receptors as benzodiazepines, but with less affinity and milder clinical effects [38]. Recently, Dong-Han et al. [39] has suggested that stimulation of the inhibitory GABA receptors can attenuate the excitatory JNK3 apoptotic signaling pathway via inhibiting the increased assembly of the GluR6-PSD-95-MLK3 signaling module in cerebral ischemia.

The neurotoxic action of rotenone involves apoptosis, which is inhibited by a broad-spectrum caspase inhibitor zVAD or a caspase-3 specific inhibitor DEVD. Furthermore, Rotenone activated the p38 and JNK but not ERK 1/2 signaling pathways. Blocking p38 or JNK signaling inhibited rotenone-induced apoptosis, supporting a pro-apoptotic role for these pathways in rotenone-induced apoptosis [40].

We previously found that rotenone has a deleterious effect on the ultra structure of neuroblastoma cells. Membranes were more loosely configured irregular in shape and fragmented with dark mitochondria compared to those under control. Since nuclear chromatin condensation or clumping is a hallmark of apoptosis, we studied the effects of Catuaba on apoptotic cells. We observed that Catuaba blocked rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells [29]. Chen et al. showed that ropinirole, a D2/D3 receptor agonist, can prevent rotenone-induced apoptosis in dopaminergic cell line SH-SY5Y through D3 receptor, blocking the rotenone-induced phosphorylation of JNK, P38 and p-c-Jun, but promoting the phosphorylation of ERK1/2 [41]. It should be noted that *Valeriana Officinalis* is a traditionally used sleep remedy, but the mechanism of action and the substances responsible for its sedative and sleep-enhancing properties are not fully understood [42]. Since no evidence exist about GABA receptors in SH-SY5Y cells, it may be hypothezised that *Valeriana officinalis* extract modulates rotenone-induced apoptosis through D2/D3 receptor.

Valeriana officinalis root is endowed with flavonoids, including those with activity on CNS [23, 43], and several works attribute strong neuroprotective properties to flavonoids [44–47]. This effect is frequently associated with antioxidative properties [48, 49]. Lignans from *Valeriana officinalis* with an antioxidative activity have also been identified [16]. In fact, the ROS formation and consequent oxidative stress by rotenone inhibition of mitochondrial complex I seems to have an important role in toxicity as induced by rotenone in this in vitro model. Furthermore, there are other evidences to strengthen this hypothesis; for example, it is suggested that *Valeriana officinalis* extract-mediated neuroprotection against amyloid toxicity may inhibit the increase in the $[Ca^{2+}]_i$ and antioxidative action [24].

The presence of flavonoids in *Valeriana officinalis* extract makes this plant attractive for the search of a daily neuroprotective product for human consumption [24]. However, it is important to consider that an extract is a mixture of different substances, so other compounds might be responsible for these effects. Candidates that may act in CNS also include volatile oils, like valeneric acid or valeranone, or the valepotriate group [15].

To conclude, it is clear that a direct correlation between in vitro results and in vivo effects cannot be utilized but our findings indicate that the extract of *Valeriana officinalis* has a cytoprotective effect on rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells.

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