

Valeriana officinalis attenuates the rotenone-induced toxicity in *Drosophila melanogaster*

Jéssie Haigert Sudati^a, Francielli Araújo Vieira^{a,1}, Sandra Sartoretto Pavin^a, Glaecir Roseni Mundstock Dias^a, Rodrigo Lopes Seeger^a, Ronaldo Golombieski^a, Margareth Linde Athayde^b, Félix Antunes Soares^a, João Batista Teixeira Rocha^{a,*}, Nilda Vargas Barbosa^{a,*}

^a Departamento de Química, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria (UFSM), Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

^b Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97100-900 Santa Maria, RS, Brazil

ARTICLE INFO

Article history:

Received 8 December 2012

Accepted 17 April 2013

Available online 29 April 2013

Keywords:

Drosophila melanogaster

Rotenone

Valeriana officinalis

Parkinson's disease

ABSTRACT

In this study, we investigated the potential protective effects of *Valeriana officinalis* (*V. officinalis*) against the toxicity induced by rotenone in *Drosophila melanogaster* (*D. melanogaster*). Adult wild-type flies were concomitantly exposed to rotenone (500 μ M) and *V. officinalis* aqueous extract (10 mg/mL) in the food during 7 days. Rotenone-fed flies had a worse performance in the negative geotaxis assay (*i.e.* climbing capability) and open-field test (*i.e.* mobility time) as well as a higher incidence of mortality when compared to control group. *V. officinalis* treatment offered protection against these detrimental effects of rotenone. In contrast, the decreased number of crossings observed in the flies exposed to rotenone was not modified by *V. officinalis*. Rotenone toxicity was also associated with a marked decrease on the total-thiol content in the homogenates and cell viability of flies, which were reduced by *V. officinalis* treatment. Indeed, rotenone exposure caused a significant increase in the mRNA expression of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and also in the tyrosine hydroxylase (TH) gene. The expression of SOD and CAT mRNAs was normalized by *V. officinalis* treatment. Our results suggest that *V. officinalis* extract was effective in reducing the toxicity induced by rotenone in *D. melanogaster* as well as confirm the utility of this model to investigate potential therapeutic strategies on movement disorders, including Parkinson disease (PD).

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, afflicting 1–2% of the population above the age of 60 years. The pathological hallmarks of PD include selective loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies in surviving dopamine neurons (Goedert et al., 1998; Cannon and Greenamyre, 2010). Lewy bodies are cytoplasmic inclusions composed mainly of alpha-synuclein, which are believed to disrupt the brain's normal functioning in PD. Although, it is well known that environmental exposures and individual genetic susceptibility may determine the onset of PD

symptoms; the precise cellular and molecular mechanism(s) responsible for the neurodegeneration processes remain still unknown. However, free radical overproduction and abnormalities in mitochondria function have emerged as critical mediators of the neuronal damage in PD (Sherer et al., 2002; Coulom and Birman, 2004; Rama Rao et al., 2007; Valko et al., 2007). Knowing that oxidative stress plays a central role in PD disease; numerous studies have been conducted to investigate the potential neuroprotective action of antioxidants in neurotoxin-based models of PD. Though several synthetic antioxidants are available, a growing trend has been targeting medicinal plants as antioxidants since herbal compounds empirically used in folk medicine are generally free of adverse effects.

The root extracts of *Valeriana officinalis* (*V. officinalis* L., Valerianaceae) have been used for centuries in popular medicine for the treatment of sleep disorders, anxiety and epilepsy (Hadley and Petry, 2003). *V. officinalis* can modulate anxiety and insomnia by interacting with different neurotransmitter systems (Malva et al., 2004; Ortiz et al., 2006; Sichardt et al., 2007; Sudati et al.,

* Corresponding author at: Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 3220 8140; fax: +55 55 3220 8978.

E-mail addresses: jbtrocha@yahoo.com.br (J.B.T. Rocha), nvbarbosa@yahoo.com.br (N.V. Barbosa).

¹ In memoriam.

2009; Del Valle-Mojica et al., 2011). However, there are only limited data on the possible protective effect of *V. officinalis* in neurodegeneration.

Regarding to PD, it is well recognized that the rotenone model of PD reproduces some important aspects of the pathology (Coulom and Birman, 2004; Cannon and Greenamyre, 2010). However, this model has limitations that are mainly related to the variability of percentage of animals that development nigrostriatal lesion and the level of lesion (Cannon and Greenamyre, 2010). Consequently, there is a need for improvement of current models and development of new experimental models. It has been amply accepted the use of the fruit fly *Drosophila melanogaster* (*D. melanogaster*) as a model to study molecular mechanisms involved in neurological diseases, including PD (Nichols, 2006; Hirth, 2010; Rand, 2010; Girish and Muralidhara, 2012). Notably, the fly has homology among five of the six genes related to PD in humans (Whitworth et al., 2006). In view of the probable advantages of the rotenone model on flies in terms of reproducibility and experimental design, we aimed to investigate the possible protective effects of *V. officinalis* on rotenone-induced toxicity in *D. melanogaster*. Specifically, we evaluated the potential of *V. officinalis* powder root in attenuating lethality, toxicity, movement disorders and some biochemical parameters of oxidative stress induced by rotenone exposure in flies.

2. Materials and methods

2.1. Chemicals

Rotenone, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ampliflu red fluorescent dye and *Horseradish peroxidase* were obtained from Sigma Chemical Company (St Louis, MO, USA).

2.2. Plant material

Powder from roots of *V. officinalis* was obtained from Bio extracts (São Paulo, Brazil). Aqueous extract was obtained after 15 min of infusion using water.

2.3. *Drosophila stock*

D. melanogaster wild-type was obtained from the National species Stock Center, Bowling Green, OH, USA. The flies were reared on agar medium (1%, w/v brewer's yeast; 2%, w/v sucrose; 1%, w/v powdered milk; 1%, w/v agar; 0.08%, w/v nipagin) at constant temperature and humidity ($23 \pm 1^\circ\text{C}$; 60% relative humidity, respectively). The flies were reared in 2.5 cm \times 6.5 cm vials containing 5 mL of medium at constant temperature, humidity (60%) and under 12 h dark/light cycle. All experiments were performed with the same strain.

2.4. Rotenone exposure and *V. officinalis* treatment

Flies (both gender, 1–2 days old) were divided into four groups: (1) control; (2) rotenone; (3) *V. officinalis* and (4) rotenone plus *V. officinalis*. Rotenone (dissolved in ethanol 98%) and *V. officinalis* (aqueous extract) were added into the food's flies at final concentration of 500 μM and 10 mg/mL, respectively. The total food medium contained a volume of 1% of ethanol, *V. officinalis* aqueous extract, rotenone or rotenone plus *V. officinalis*. Two controls were used (with and without ethanol). However, only the ethanol control is showed in the results because there was no statistical difference between these groups in all parameters evaluated. The flies were exposed to treatments during 7 days (Moreira et al., 2012) and the vials containing flies were

maintained in an incubator at $23 \pm 1^\circ\text{C}$ before being used for different assays. The concentration of rotenone used in this protocol was based on a survival curve (0.1, 0.5 and 1 mM), and corresponds to the concentration that caused approximately 50% death of the flies after 7 days of exposure (data not shown). The choice of *V. officinalis* aqueous extract concentration was based on previous observations, which showed that *V. officinalis* aqueous extract in the range of 5–20 mg/mL did not cause overt signals of toxicity in flies (data not shown).

2.5. Valeric acid, gallic acid, ellagic acid and quercetin quantification

High performance liquid chromatography (HPLC-DAD) of aqueous extract of *V. officinalis* was performed with the HPLC system (Shimadzu, Kyoto, Japan), according to a previous work from our group (Sudati et al., 2009). Briefly, stock solutions of valeric acid, gallic acid, ellagic acid and quercetin standard reference were prepared in the HPLC mobile phase at a concentration range of 3.12–50.0 $\mu\text{g/mL}$. Quantification was carried out by the integration of the peaks using external standard method at 220 nm for valeric acid, 257 nm for gallic acid, 325 nm for ellagic acid and 356 for quercetin. The flow rate was 1.5 mL/min and the injection volume was 10 μL . The chromatographic peaks were confirmed by comparing their retention time and DAD-UV spectra (200–400 nm) and with those of the reference standards. All chromatographic operations were carried out at ambient temperature and in triplicate.

2.6. In vivo assays

2.6.1. Survival rate

The survival rate was evaluated by counting daily of the number of living flies until the end of the experimental period (7 days). Around 150 flies per group were included in the survival data and the total number of flies represents the sum of three independent experiments (50 flies/each treatment repetition).

2.6.2. Negative geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously by Feany and Bender (2000). The flies (both gender) were sorted under a brief ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 5 flies each). After the recovery from cold exposure (approximately 20 min) the flies were gently tapped to the bottom of the column. The flies that reached the top of the column (6 cm) and the flies that remained at the bottom were counted separately during 6 s. The scores represent the mean of the numbers of flies at the top (n_{top}) as percentage of the total number of flies (n_{tot}). Around 15 flies per group were included for the negative geotaxis data (total of 45 flies). This value represents the sum of three independent experiments.

2.6.3. Open-field

Open-field task was performed according to the method described by Hirth (2010), with some modifications. Each wild-type fly was kept in an arena divided by squares (1 cm \times 1 cm) measuring 9 cm of diameter, which can be covered by petri dish. The fly's activity and movement were recorded with a video camera and the distance travelled was recorded by the resulting trajectory during a given time-window (60 s), which was calculated throughout the number of squares crossed by each single fly analyzed. Video-assisted movement tracking records locomotor behavior allows the quantification of immobility (time spends without locomotion) and distance travelled (number of square crossed). Around 15 flies were included for each treatment performed and the total number of flies contained in the open-field

data (45 per group) represents the sum of three independent experiments.

2.7. Ex vivo assays

2.7.1. Homogenate preparation

About 20 flies per group were immobilized by chilling on ice and then decanted into a chilled mortar. Whole body of flies was manually homogenized in ice-cold Tris/HCl buffer (pH 7.4, 0.1 M), 1:10 (flies/volume (μL)). The homogenate was filtered through sieve with nylon mesh (pore size/10 mm), centrifuged at $3000 \times g$ for 3 min at 4°C , and the supernatant was used for biochemical assays (20 flies/200 μL).

2.7.2. RNA Isolation and analysis of mRNA expression by q-PCR (quantitative real-time PCR)

Approximately 2 μg of total RNA from 20 flies per group was extracted using Trizol[®] reagent (Invitrogen[®]) according to the manufacturer's suggested protocol. The primers sequences for genes of Catalase (CAT), superoxide dismutase (SOD) and tyrosine hydroxylase (TH) were designed. Gene specific primer sequences were based on published sequences in GenBank Overview (<http://www.ncbi.nlm.nih.gov/genbank/>) designed with Primer3 program version 0.4.0 (<http://frodo.wi.mit.edu/primer3/>) and custom made by Invitrogen[®] (Table 1). After quantification, total RNA was treated with DNase I (Invitrogen[®]). The cDNA was synthesised with M-MLV reverse transcriptase enzyme and random primer (Invitrogen) according to the manufacturer's suggested protocol. Quantitative real-time polymerase chain reaction was performed in 20 μL PCR mixture containing 1 μL RT product (cDNAs) as template, $1 \times$ PCR buffer, 25 μM dNTPs, 0.2 μM of each primer, 1.5–2.5 mM MgCl_2 , 0.1 \times SYBR Green I (Molecular Probes[®]) and 1 U Taq DNA polymerase (Invitrogen[®]) (Golombieski et al., 2008). The thermal cycle was carried in a Thermocycler StepOne Plus (Applied Biosystems) and the protocol used was the following: activation of the Taq DNA polymerase 95°C for 5 min followed of 40 cycles of 15 s at 95°C , 15 s at 60°C and 25 s at 72°C . SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems), and the CT value for each sample was calculated and reported using the $2^{-\Delta\Delta\text{CT}}$ method described by Livak and Schmittgen (2001). For each well, analyzed in quadruplicate, a ΔCT value was obtained by subtracting the actin and GPDH CT value from the C T value of the interest gene. The ΔCT mean value obtained from the control group of each gene was used to calculate the $\Delta\Delta\text{CT}$ of the respective gene ($2^{-\Delta\Delta\text{CT}}$). All experiments were calculated in relation to both gene controls: tubulin and GPDH. Three independent experiments were performed with 20 flies collected and qPCR quantification was performed in quadruplicate.

Table 1
Sequences of qPCR primers.

Primer	Sequence
Sod LEFT	5' GGAGTCGGTGATGTGACCT 3'
Sod RIGHT	5' GTTCGGTGACAACACCAATG 3'
Catalase LEFT	5' ACCAGGGCATCAAGAATCTG 3'
Catalase RIGHT	5' AACTTCTTGGCCTGCTCGTA 3'
TH LEFT	5' TACAAGCCACGGAGTTCCT 3'
TH RIGHT	5' GTCAGGCCAGACTTCAAAGC 3'
GPDH LEFT	5' ATGGAGATGATTCCGCTTCGT 3'
GPDH RIG HT	5' GCTCCTCAATGGTTTTTCCA 3'
Tubulin LEFT	5' ACCAATGCAAGAAGCCCTTG 3'
Tubulin RIGHT	5' ATCCCAACAACCTGAAGAC 3'

2.7.3. Cell viability evaluation

Cell viability was evaluated by dehydrogenase activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This analysis was performed in whole body of flies by two different manners (with and without homogenate procedure), according to the method described by Babot et al. (2005). The ratio values were standardized per protein content and expressed as percentage in relation to the control.

2.7.4. Thiol determination

Total and non-protein thiol contents were estimated based on a spectrophotometric method using Ellman's reagent, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). For non-protein thiol, the homogenate was precipitated with TCA 10% followed by centrifugation at $3000 \times g$ for 3 min at 4°C . The supernatant samples were measured spectrophotometrically at 412 nm. A standard curve was constructed for each measurement using GSH. Thiol levels were expressed as percentage in relation to the control.

2.7.5. Isolation of mitochondria

After the treatment (described previously), mitochondria from *D. melanogaster* were isolated according to Miwa and Brand (2005), with minor modifications. Briefly, about 50 flies were immobilized by chilling on ice, and gently pressed with a pestle in a chilled mortar containing a little isolation medium (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 1% (w/v) bovine serum albumin, adjusted to pH 7.4 at 4°C) then passed through absorbent muslin and collected into a tube (1.5 mL) and immediately centrifuged at $150 \times g$ for 3 min at 4°C . The supernatant was passed through one layer of muslin and recentrifuged at $9000 \times g$ for 10 min. The supernatant was discarded and the pellet was carefully resuspended in isolation medium to give about 30 mg protein/mL. The detailed characteristics of mitochondria prepared on this way were previously reported by Miwa et al. (2003). The experiments were started as soon as possible after the isolation procedure.

2.7.6. Measurement of mitochondrial H_2O_2 production

H_2O_2 released from mitochondria was detected using the Ampliflu red fluorescent dye, which reacts with H_2O_2 in the presence of *Horseradish peroxidase*, producing highly fluorescent resourcing. *Horseradish peroxidase* (0.2 U/mL) and Ampliflu red reagent (1 μM) were added to the assay medium and then mitochondria (≈ 0.1 mg/mL) were applied. H_2O_2 formation was initiated by the addition of pyruvate (5 mM) and fluorescence was detected at 30°C in a Shimadzu spectrophotometer. The excitation and emission fluorescent wavelengths were 550 and 585 nm, slit (3 and 5) respectively. The positive control signal was produced by the addition of known amounts of H_2O_2 at the end of each experiment.

2.7.7. Protein determination

Protein concentrations in the whole body homogenates were determined by the method of comassie blue (Bradford, 1976), using bovine serum albumin as the standard.

2.8. Statistical analyses

Behavior and survival data were evaluated by nonparametric methods because Levene's test indicated the absence of Homogeneity of Variance. Behavior parameters were analyzed using Kruskal–Wallis (kw) followed by Dunn's multiple comparisons test when appropriate and survival by the χ^2 method and Fisher's exact test (ESTATISTIC Module Switcher Program). All other results were analyzed by two-way ANOVA followed by Duncan multiple range test when appropriate. Differences between groups were considered significant when $p < 0.05$. Data of non-parametric analysis are represented as medians and ranges (interquartile interval); and

data of parametric analysis as means and S.E.M. Graphics were created using GraphPad Prisma 4.0.

3. Results

3.1. HPLC analyses

HPLC fingerprinting of the aqueous extract of *V. officinalis* (10 mg/mL) showed an elution diagram when the peaks were grouped in regions based on the UV absorption profile (Fig. 1). The chromatograms of aqueous extract revealed a peak 2 with a retention time (r.t.) at 2.55 min corresponding to valeric acid ($y = 668.6x + 3.361$, $r = 0.9960$) in the concentration of 2.29 mg/mL. Indeed, it was found a peak 3 (r.t. = 4.39 min), corresponding to ellagic acid ($y = 328.4x + 2.196$, $r = 0.9997$) at concentration of 0.71 mg/mL; a peak 4 (r.t. = 5.32 min) corresponding to gallic acid ($y = 245.7x + 1.480$, $r = 0.9998$) at concentration of 0.47 mg/mL and a peak 5 (r.t. = 8.66 min) supporting the presence of quercetin ($y = 258.3x + 1.537$, $r = 0.9999$) at concentration of 0.11 mg/mL.

3.2. Effect of *V. officinalis* on survival rate of flies exposed to rotenone

Data of Fig. 2 show that rotenone exposure caused a time dependent increase in the percentage of deaths until the end of experimental period when compared to the control group. The mortality rate was lower in the flies treated simultaneously with *V. officinalis*. *V. officinalis* alone did not alter the survival of the flies when compared to the control group (Fig. 2).

3.3. Effect of *V. officinalis* on negative geotaxis assay in the flies exposed to rotenone

Dunn's multiple comparisons test revealed that the climbing behavior was impaired in the flies exposed to rotenone, when compared to control flies ($kw = 10.77$; $p = 0.029$; Fig. 3). This effect of rotenone was abolished by *V. officinalis* treatment. Statistical analysis also showed that there was not significant difference in this parameter between *V. officinalis* alone and control group ($kw = 10.77$; $p > 0.05$).

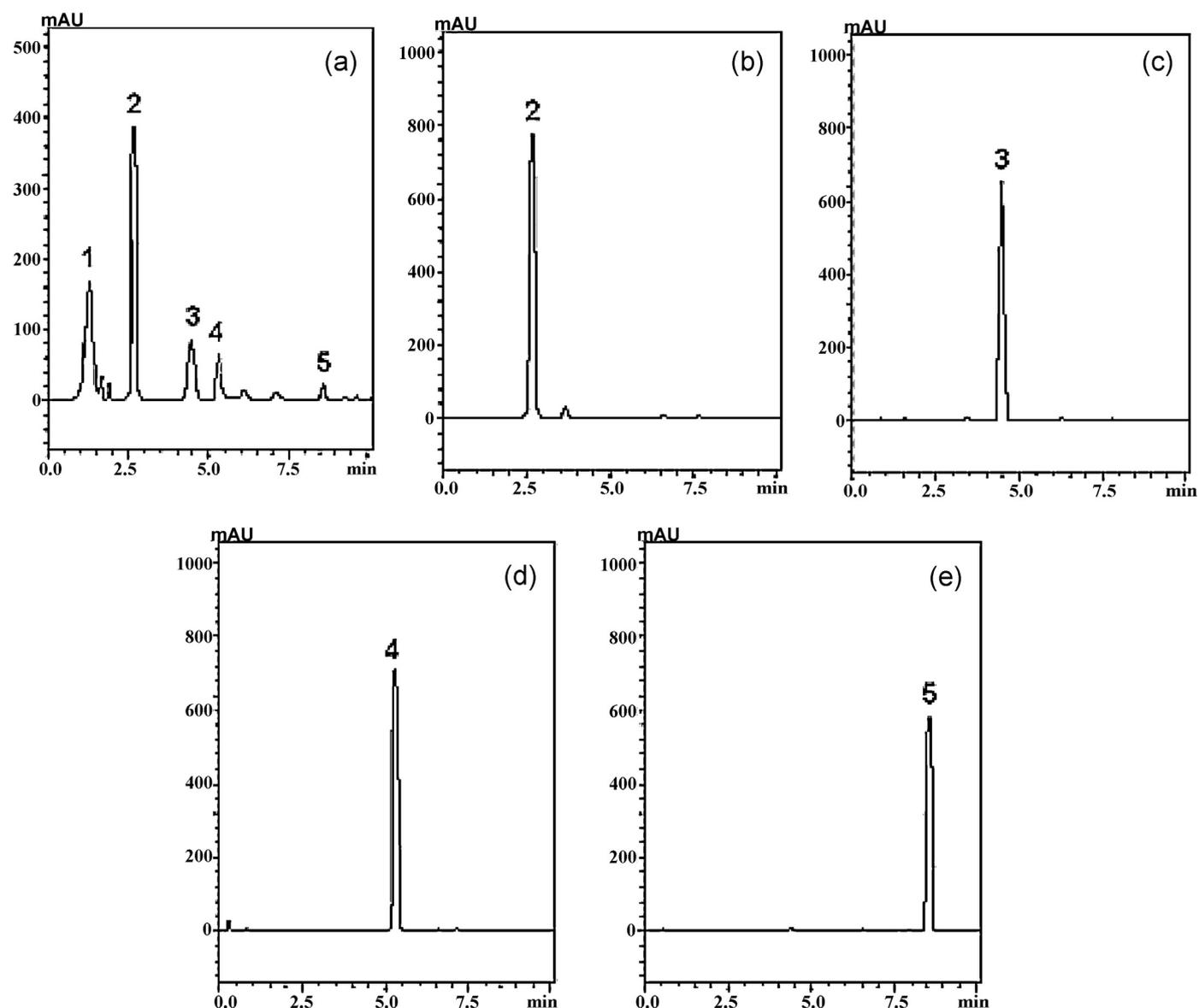


Fig. 1. (A) High performance liquid chromatography of *V. officinalis* aqueous extract; peak 1: represents an unknown peak, peak 2: corresponds to valeric acid, peak 3: ellagic acid, peak 4: gallic acid and peak 5: quercetin. (B) Representative chromatogram of valeric acid standard (peak 2), (C) chromatogram of ellagic acid standard (peak 3), (D) chromatogram of acid gallic standard (peak 4) and (E) chromatogram of quercetin standard. Chromatographic conditions are described in Section 2.

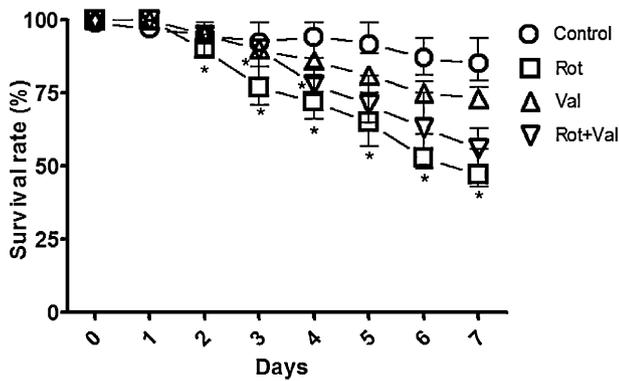


Fig. 2. Effect of *V. officinalis* on survival rate of flies exposed to rotenone. Data were collected every 24 h for each group during 7 days. The numbers of surviving flies are represented as % of control. The total number of flies (150 per group) represents the sum of three independent experiments. *Significant difference in relation to the control group (Fischer's exact probability test). Values are expressed as median and range (interquartile interval).

3.4. Effect of *V. officinalis* on open-field test in the flies exposed to rotenone

In open-field evaluation, Kruskal–Wallis test followed by Dunn's multiple comparisons test revealed that the flies exposed to rotenone had a significant decrease in the number of crossings ($kw = 50.66$; $p = 0.0001$) when compared to the control group and that this effect was not modified by concomitant treatment with *V. officinalis* ($kw = 50.66$; $p > 0.05$) (Fig. 4A). No significant difference was observed between *V. officinalis* and control group in this parameter ($kw = 50.66$; $p > 0.05$).

Dunn's multiple comparisons test revealed that the immobility, evaluated by time spent without moving, was significantly increased in the flies exposed to rotenone ($kw = 17.90$; $p = 0.0013$) when compared to control group (Fig. 4B). This effect of rotenone was blunted by *V. officinalis* treatment. Kruskal–Wallis test also indicated that there was no significant difference between *V. officinalis* and control group in this evaluation ($kw = 17.90$; $p > 0.05$).

3.5. mRNA expression of SOD, CAT and TH genes

Two-way ANOVA followed by *post hoc* comparisons showed that flies exposed to rotenone had a significant increase in the mRNA expression of SOD (Fig. 5A) and CAT (Fig. 5B), when

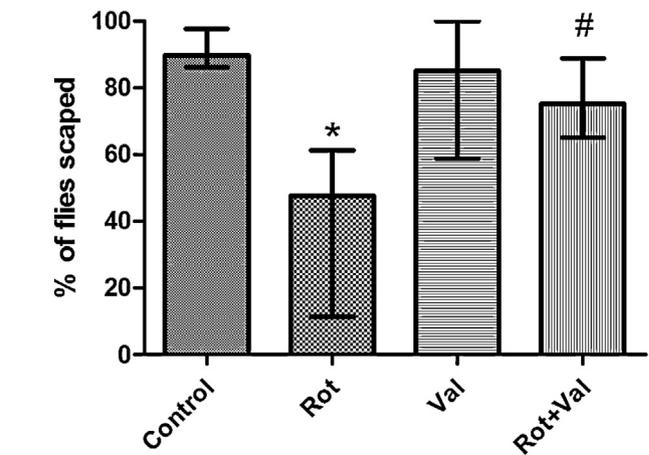
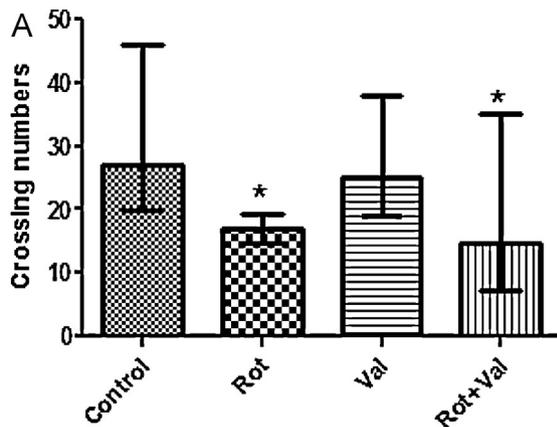


Fig. 3. Effect of *V. officinalis* on geotaxis response (climbing) of flies exposed to rotenone during 7 days. The total number of flies (≈ 45 per group) represents the sum of three independent experiments (15 flies/each analysis). Values are expressed as median and range (interquartile interval). *Significant difference in relation to the control group; #Significant difference between Rot and Rot + Val groups (Kruskal–Wallis test followed Dunn' multiple comparisons test, $p < 0.05$).

compared to the values found in the control group. These effects induced by rotenone exposure on antioxidant genes were normalized by *V. officinalis* treatment. The mRNA expression of CAT was increased in the flies treated with *V. officinalis* alone (Fig. 5B). Statistical analysis revealed a significant main effect of rotenone [$F(1,8) = 13.65$, $p < 0.0061$] on SOD and *V. officinalis* on CAT mRNA expression [$F(1,8) = 5.57$, $p < 0.046$]. Indeed, a significant rotenone \times *V. officinalis* interaction was verified in both SOD [$F(1,8) = 13.55$, $p < 0.006$] and CAT [$F(1,8) = 26.7$, $p < 0.0009$] mRNA expression.

Rotenone exposure also caused an increase on mRNA expression of TH, which was not restored by concomitant treatment with *V. officinalis* (Fig. 5C). In this parameter, there was a significant main effect of *V. officinalis* [$F(1,8) = 32.65$, $p < 0.0004$]. No significant interaction between rotenone and *V. officinalis* was observed on mRNA TH expression [$F(1,8) = 1.574.90$, $p < 0.038$].

3.6. Effect of *V. officinalis* on MTT reduction in homogenate and body region of flies exposed to rotenone

Two-way ANOVA followed by *post hoc* comparisons showed that rotenone exposure caused a significant decrease on MTT

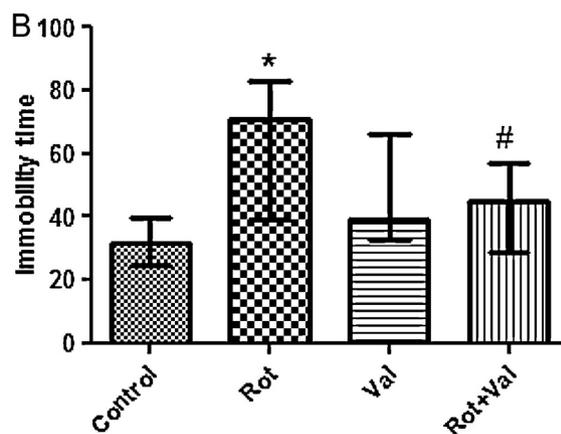


Fig. 4. Effect of *V. officinalis* on locomotor and exploratory activities evaluated by open-field of the flies exposed to rotenone. Two parameters were analyzed: (A) exploratory activity (evaluated by counting the crossing numbers) and (B) immobility time. The total number of flies (≈ 50 per group) represents the sum of three independent experiments. Values are expressed as median and range (interquartile interval). *Significant difference in relation to the control group; #Significant difference between Rot and Rot + Val groups (Kruskal–Wallis test followed Dunn' multiple comparisons test, $p < 0.05$).

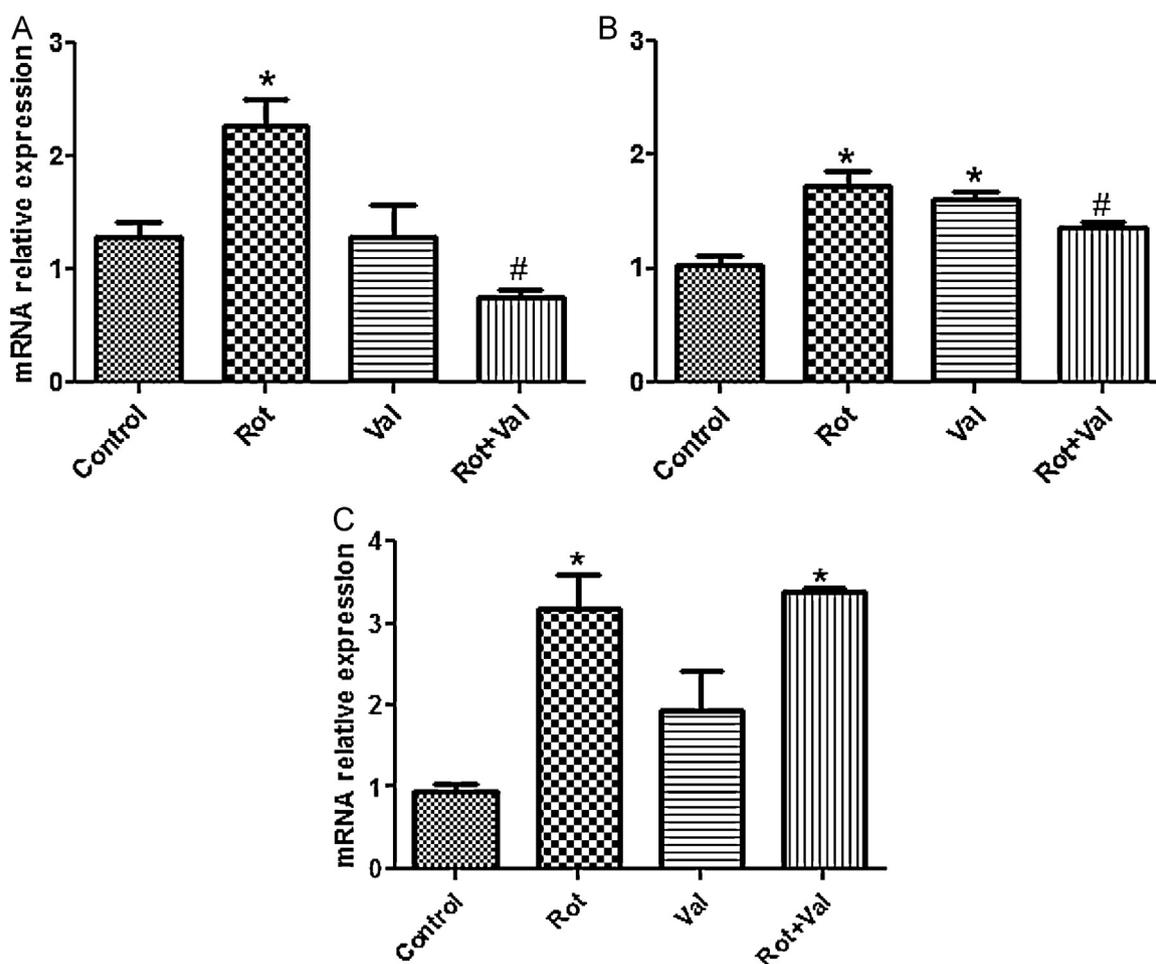


Fig. 5. qPCR relative gene expression of superoxide dismutase (SOD) graph A, catalase (CAT), graph B and tyrosine hydroxylase (TH), graph C. Results are expressed as mean \pm S.E.M ($n = 3$). The experiment was done from 3 independent homogenization of 20 flies each, and qPCR quantification was performed in quadruplicate. *Significant difference in relation to control group; #significant difference between Rot and Rot + Val groups ($p < 0.05$, two-way ANOVA followed Duncan multiple range test).

reduction in the fly homogenates and that this effect was blunted by *V. officinalis* treatment (Fig. 6). Indeed, statistical analysis revealed a main effect of *V. officinalis* [$F(1,20) = 4.90$, $p < 0.038$]. Rotenone \times *V. officinalis* interaction was not significant on MTT reduction [$F(1,20) = 2.21$, $p < 0.15$].

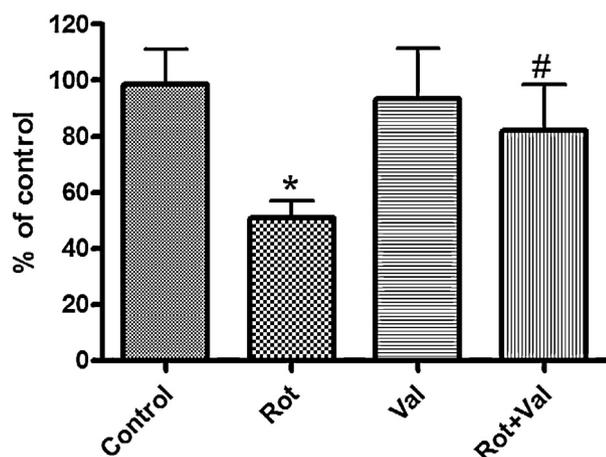


Fig. 6. Effect of *V. officinalis* on cell viability in homogenate of flies exposed to rotenone. *Significant difference in relation to control group. Values are expressed as mean \pm S.E.M ($n = 9$). #Significant difference between Rot and Rot + Val groups ($p < 0.05$, two-way ANOVA followed by Duncan multiple range test).

To check if the homogenization procedure could influence these results, we performed the same assay without homogenizing the flies. As observed with homogenates, MTT reduction in entire flies was decreased by rotenone and this effect was abolished by *V. officinalis* treatment (data not shown).

3.7. Effect of *V. officinalis* on thiol content in homogenate of flies exposed to rotenone

Two-way ANOVA followed by *post hoc* comparisons showed that rotenone exposure diminished significantly the total-thiol content of the flies when compared to the control group (Fig. 7A). *V. officinalis* treatment blunted the rotenone-induced decrease on the total-thiol content in the flies. Statistical analysis also indicated a significant main effect of *V. officinalis* on thiol levels [$F(1,12) = 10.48$, $p < 0.007$]. No significant main effect of rotenone [$F(1,12) = 0.06$, $p < 0.98$] and/or rotenone \times *V. officinalis* interaction [$F(1,12) = 0.25$, $p < 0.628$] was found in this parameter.

No significant difference was observed among the groups on the non-protein thiols levels (Fig. 7B).

3.8. Effect of *V. officinalis* on H_2O_2 mitochondrial production in mitochondria of flies exposed to rotenone

Two-way ANOVA followed by *post hoc* comparisons showed that H_2O_2 mitochondrial formation was increased in the flies exposed to rotenone (line d and inset columns) compared to

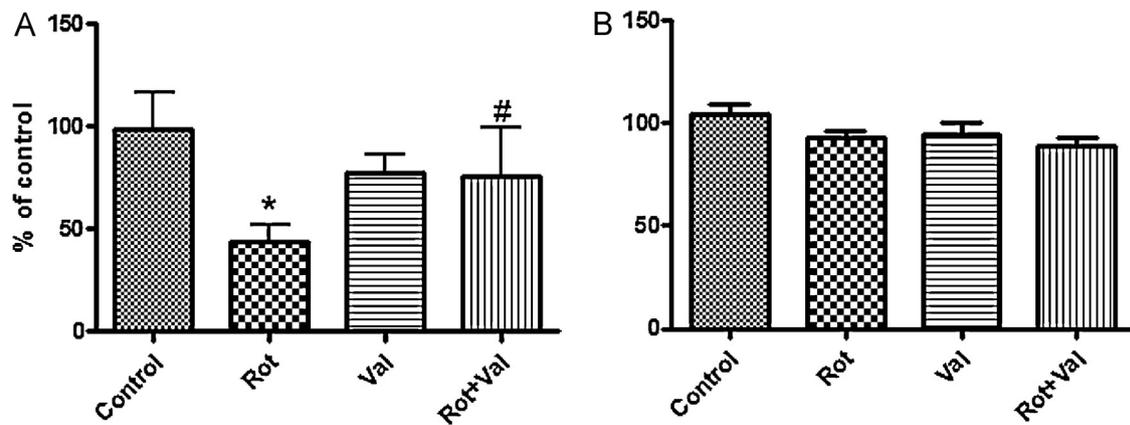


Fig. 7. Effect of *V. officinalis* on total-thiol (A) and non-protein thiol (B) content in homogenate of flies exposed to rotenone. *Significant difference in relation to control group; #significant difference between Rot and Rot + Val groups. Values are expressed as mean \pm S.E.M ($n = 9$) ($p < 0.05$, two-way ANOVA followed by Duncan multiple range test).

control group (line b; $p < 0.05$). The rotenone-induced over production of H_2O_2 was reduced by *V. officinalis* treatment (line e and inset, Fig. 8). Indeed, a significant main effect of *V. officinalis* [$F(1,12) = 10.44$, $p < 0.007$] and rotenone \times *V. officinalis* interaction [$F(1,12) = 4.75$, $p < 0.05$] was verified on H_2O_2 mitochondrial levels.

4. Discussion

Currently, *D. melanogaster* has been used as an alternative animal model for screening of natural therapeutic agents for the treatment of neurodegenerative diseases, including PD (Hosamani and Muralidhara, 2009; Jeibmann and Paulus, 2009). With this in mind, the present study was delineated to evaluate the possible beneficial effects of *V. officinalis* against the toxicity related to the rotenone exposure in *D. melanogaster*. We have found that the aqueous extract from roots of *V. officinalis* was effective in reducing motor impairments and some oxidative stress parameters mediated by rotenone.

In our experimental protocol, exposure of *D. melanogaster* to rotenone reproduced key aspects of PD, for instance locomotor deficits. In fact, the flies exposed to rotenone exhibited impairment in negative geotaxis (climbing) and in the open-field test (crossing numbers and immobility time). Interestingly, most of these

movement disturbances were ameliorated by *V. officinalis* treatment. Indeed, *V. officinalis* reduced the mortality rate of the flies exposed to rotenone. These beneficial effects of *V. officinalis* observed here are in accordance with the data of a recent study where the plant was able in avoiding oral movement deficits induced by reserpine in rats (Pereira et al., 2011).

A growing body of evidence suggests that the exposure to neurotoxins such as rotenone, paraquat and/or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can lead to the PD like pathology and symptoms (Valko et al., 2007; Cicchetti et al., 2009; Goldman et al., 2012). Although, little is known about the precise mechanism action of these agents in *D. melanogaster*; the involvement of mitochondrial complex I, is well supported by several experimental studies (Cicchetti et al., 2009; Norenberg and Rao, 2007). Indeed, there is clue that these pesticides can induce Lewy bodies formation, dopaminergic neurodegeneration, decrease in striatal dopamine levels and disturbances in GSH homeostasis (Garrido et al., 2011). Alterations in gene expression like tyrosine hydroxylase, alpha-synuclein, PARKIN, PINK1 or DJ-1, which are involved in the sporadic forms of PD, have also been associated with the toxicity of rotenone and MPTP (Rajput and Rajput, 2007). Thus, it is widely accepted that the effects mediated by rotenone encompass oxidative stress linked with mitochondrial dysfunction and reactive oxygen species (ROS) overproduction (Cicchetti et al., 2009).

Here we observed that rotenone exposure increased the mRNA expression of antioxidant enzymes (SOD and CAT) and TH, which may represent a compensatory response to oxidative insults. Here we have not determined concomitantly the levels and/or activity of respective enzymes; and this fact could limit an analysis more detailed of our results. However, accompanied or not by changes in the activity of enzymes, it is reasonable assume that rotenone influences the transcription of SOD and CAT enzymes genes. Indeed, rotenone reduced significantly the cell viability and the total-thiol content, without changing the non-protein thiol levels in the flies. Taken together, these data suggest a possible implication of oxidative stress on the rotenone-based model with *D. melanogaster*.

It is well established that *V. officinalis* is a medicinal plant effective in improving anxiety and sleep disorders (Hadley and Petry, 2003). However, few investigations have evaluated the effects of *V. officinalis* in experimental neurodegenerative diseases. In fact, we found only one study in the literature pointing a cytoprotective role for *V. officinalis* extracts in an *in vitro* model of PD (Oliveira et al., 2009). In this sense, our data show, for the first time, the effectiveness of *V. officinalis* in reducing the deleterious effects of rotenone in both behavior and oxidative stress

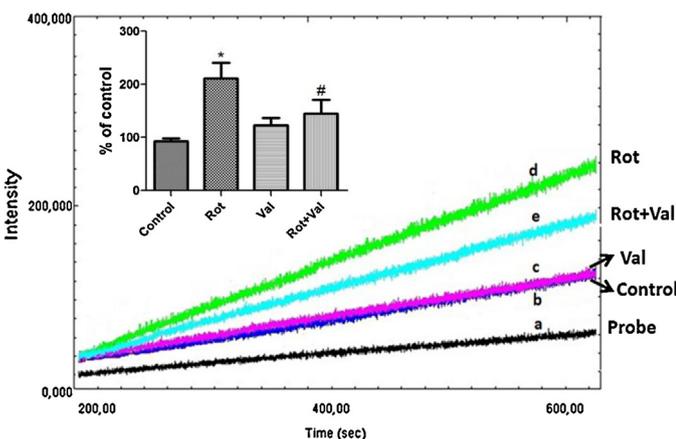


Fig. 8. Hydrogen peroxide (H_2O_2) production of isolated mitochondria from *D. melanogaster*. (A) probe alone, (B) control, (C) Val, (D) Rot and (E) Rot + Val. *Significant difference in relation to control group; #significant difference between Rot and Rot + Val groups. Lines correspond to a randomly representative graphic. Values are expressed as mean \pm S.E.M ($n = 3$) ($p < 0.05$, two-way ANOVA followed by Duncan multiple range test).

parameters after *in vivo* exposure of *D. melanogaster*. It is reasonable to assume that the protection offered by *V. officinalis* in behavior and biochemical parameters is, at least in part, associated with antioxidant properties of plant due to its phenolic and flavonoid constituents. Corroborating this idea, there is evidence that *V. officinalis* tincture is effective in inhibiting thiobarbituric acid reactive substances production and deoxyribose degradation stimulated by different pro-oxidants in brain rats (Sudati et al., 2009).

Although our study did not encompass the neuroactive role of *V. officinalis* in terms of mechanisms, it is important to mention here that both valerianic acid (a major constituent of Valerian root extracts) and herbal extracts of *V. officinalis* may enhance GABA responses (Neuhaus et al., 2008; Tariq and Pulisetty, 2008). In PD and its animal models, it is believed that GABA signaling is critical for the function of the basal ganglia given its ability in modulating dopaminergic neurons (Denora et al., 2012). In accordance, studies have showed that the severity of tremor in PD patients is correlated with abnormalities found in GABA receptor density and binding (Gironell, 2007; Gironell et al., 2012). Specifically with *D. melanogaster*, a recent study demonstrated the participation of GABA_B receptor in the etiology of PD in a model using transgenic flies carrying the human gene for alpha synuclein (Hillman et al., 2012). Consequently, it is plausible suppose that the gabaergic action of *V. officinalis* could be contributing to its pharmacological effects on the rotenone model of PD in *D. melanogaster*. Supporting this proposal, current literature findings have pointed gabaergic agonists for dopamine replacement therapy (Leal et al., 2004; Denora et al., 2012; Hillman et al., 2012).

In summary, the results of present study show that *V. officinalis* extract is efficacious in reducing the toxicity induced by rotenone in *D. melanogaster* as well as confirm the utility of this model to investigate therapeutic strategies that may be promising in treatment of neurodegenerative diseases. However, additional studies about the neurological pathways involved on the protective role of *V. officinalis* in PD will be needed. Besides, epidemiological researches should be performed to determine whether the use of *V. officinalis* in folk medicine is associated or not with a decrease in the incidence of PD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The financial support by FINEP Research Grant “Rede Instituto Brasileiro de Neurociência (IBN-Net)” #01.06.0842-00, DECIT/SCTIE-MS-FAPERGS/Pronex, Pronem, #11/2029-1, FAPERGS/ARD 03/2012/PQG, CAPES/SAUX, CAPES/NOVOS TALENTOS, VITAE Foundation, INCT-CNPq-Excitotoxicity, DECIT/SCTIE-MS and Neuroprotection and CNPq is gratefully acknowledged. J.B.T.R, F.A.S and N.B.V.B are the recipients of CNPq fellowships. Sincere posthumous thanks to our *in memoriam* friends and research group labmates: Bruna Karoline Occai, Kelly Ane Azzolin, Rafael Ferreira Dias and especially to Francielli Araújo Vieira, a co-author of this article.

References

- Babot Z, Cristofol R, Sunol C. Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on GABA receptors and niflumic acid-sensitive chloride channels. *Eur J Neurosci* 2005;21:103–12.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;72:248–54.
- Cannon JR, Greenamyre JT. Neurotoxic *in vivo* models of Parkinson's disease recent advances. *Prog Brain Res* 2010;184:17–33.
- Cicchetti F, Drouin-Ouellet J, Gross RE. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? *Trends Pharmacol Sci* 2009;30:475–83.
- Coulom H, Birman S. Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. *J Neurosci* 2004;24:10993–8.
- Del Valle-Mojica LM, Cordero-Hernandez JM, Gonzalez-Medina G, Ramos-Valdez I, Berrios-Cartagena N, Torres-Hernandez BA, et al. Aqueous and ethanolic *Valeriana officinalis* extracts change the binding of ligands to glutamate receptors. *Evid Based Complement Alternat Med* 2011;2011:891819 7 pp..
- Denora N, Cassano T, Laquintana V, Lopalco A, Trapani A, Cimmino CS, et al. Novel codrugs with GABAergic activity for dopamine delivery in the brain. *Int J Pharm* 2012;437:221–31.
- Ellman GL. Tissue sulphydryl groups. *Arch Biochem Biophys* 1959;82:70–7.
- Feany MB, Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* 2000;404:394–8.
- Garrido M, Tereshchenko Y, Zhevtsova Z, Taschenberger G, Bahr M, Kugler S. Glutathione depletion and overproduction both initiate degeneration of nigral dopaminergic neurons. *Acta Neuropathol* 2011;121:475–85.
- Girish C, Muralidhara. Propensity of *Selaginella delicatula* aqueous extract to offset rotenone-induced oxidative dysfunctions and neurotoxicity in *Drosophila melanogaster*: implications for Parkinson's disease. *Neurotoxicology* 2012;33:444–56.
- Gironell A. Therapeutic strategies for essential tremor. *Med Clin (Barc)* 2007;129:632–7.
- Gironell A, Ribosa-Nogue R, Pagonabarraga J. Withdrawal of visual feedback in essential tremor. *Parkinsonism Relat Disord* 2012;18:402–3 author reply 4.
- Goedert M, Spillantini MG, Davies SW. Filamentous nerve cell inclusions in neurodegenerative diseases. *Curr Opin Neurobiol* 1998;8:619–32.
- Goldman SM, Quinlan PJ, Ross GW, Marras C, Meng C, Bhudhikanok GS, et al. Solvent exposures and Parkinson disease risk in twins. *Ann Neurol* 2012;71:776–84.
- Golombieski RM, Graichen DA, Pivetta LA, Nogueira CW, Loreto EL, Rocha JB. Diphenyl diselenide [(PhSe)₂] inhibits *Drosophila melanogaster* delta-aminolevulinic dehydratase (delta-ALA-D) gene transcription and enzyme activity. *Comp Biochem Physiol C Toxicol Pharmacol* 2008;147:198–204.
- Hadley S, Petry JJ. Valerian. *Am Fam Phys* 2003;67:1755–8.
- Hillman R, Sinani J, Pendleton R. The role of the GABA(B) receptor and calcium channels in a *Drosophila* model of Parkinson's Disease. *Neurosci Lett* 2012;516:167–70.
- Hirth F. *Drosophila melanogaster* in the study of human neurodegeneration. *CNS Neurol Disord Drug Targets* 2010;9:504–23.
- Hosamani R, Muralidhara. Neuroprotective efficacy of *Bacopa monnieri* against rotenone induced oxidative stress and neurotoxicity in *Drosophila melanogaster*. *Neurotoxicology* 2009;30:977–85.
- Jeibmann A, Paulus W. *Drosophila melanogaster* as a model organism of brain diseases. *Int J Mol Sci* 2009;10:407–40.
- Leal SM, Kumar N, Neckameyer WS. GABAergic modulation of motor-driven behaviors in juvenile *Drosophila* and evidence for a nonbehavioral role for GABA transport. *J Neurobiol* 2004;61:189–208.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} method. *Methods* 2001;25:402–8.
- Malva JO, Santos S, Macedo T. Neuroprotective properties of *Valeriana officinalis* extracts. *Neurotox Res* 2004;6:131–40.
- Miwa S, St-Pierre J, Partridge L, Brand MD. Superoxide and hydrogen peroxide production by *Drosophila* mitochondria. *Free Radic Biol Med* 2003;35:938–48.
- Miwa S, Brand MD. The topology of superoxide production by complex III and glycerol 3-phosphate dehydrogenase in *Drosophila* mitochondria. *Biochim Biophys Acta* 2005;1709:214–9.
- Moreira CG, Barbiero JK, Ariza D, Dombrowski PA, Sabioni P, Bortolanza M, et al. Behavioral, neurochemical and histological alterations promoted by bilateral intranigral rotenone administration: a new approach for an old neurotoxin. *Neurotox Res* 2012;21:291–301.
- Neuhaus W, Trauner G, Gruber D, Oelzant S, Klepal W, Kopp B, et al. Transport of a GABA_A receptor modulator and its derivatives from *Valeriana officinalis* L. s. l. across an *in vitro* cell culture model of the blood–brain barrier. *Planta Med* 2008;74:1338–44.
- Nichols CD. *Drosophila melanogaster* neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. *Pharmacol Ther* 2006;112:677–700.
- Norenberg MD, Rao KV. The mitochondrial permeability transition in neurologic disease. *Neurochem Int* 2007;50:983–97.
- Oliveira DM, Barreto G, De Andrade DEV, Saraceno E, Aon-Bertolino L, Capani F, et al. Cytoprotective effect of *Valeriana officinalis* extract on an *in vitro* experimental model of Parkinson disease. *Neurochem Res* 2009;34.
- Ortiz JG, Rassi N, Maldonado PM, Gonzalez-Cabrera S, Ramos I. Commercial valerian interactions with [3H]Flunitrazepam and [3H]MK-801 binding to rat synaptic membranes. *Phytother Res* 2006;20:794–8.
- Pereira RP, Fachineto R, de Souza Prestes A, Wagner C, Sudati JH, Boligon AA, et al. *Valeriana officinalis* ameliorates vacuole chewing movements induced by reserpine in rats. *J Neural Transm* 2011;118:1547–57.
- Rajput A, Rajput AH. Old age and Parkinson's disease. *Handb Clin Neurol* 2007;84:427–44.
- Rama Rao KV, Reddy PV, Hazell AS, Norenberg MD. Manganese induces cell swelling in cultured astrocytes. *Neurotoxicology* 2007;28:807–12.
- Rand MD. *Drosophila* neurotoxicology: the growing potential for *Drosophila* in neurotoxicology. *Neurotoxicol Teratol* 2010;32:74–83.
- Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, et al. An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci* 2002;22:7006–15.

- Sichardt K, Vissienon Z, Koetter U, Brattstrom A, Nieber K. Modulation of postsynaptic potentials in rat cortical neurons by valerian extracts macerated with different alcohols: involvement of adenosine A(1)- and GABA(A)-receptors. *Phytother Res* 2007;21:932–7.
- Sudati JH, Fachinnetto R, Pereira RP, Boligon AA, Athayde ML, Soares FA, et al. In vitro antioxidant activity of *Valeriana officinalis* against different neurotoxic agents. *Neurochem Res* 2009;34:1372–9.
- Tariq SH, Pulisetty S. Pharmacotherapy for insomnia. *Clin Geriatr Med* 2008;24:93–105 vii.
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44–84.
- Whitworth AJ, Wes PD, Pallanck LJ. *Drosophila* models pioneer a new approach to drug discovery for Parkinson's disease. *Drug Discov Today* 2006;11:119–26.