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ORIGINAL ARTICLE

# KM-34, a Novel Antioxidant Compound, Protects against 6-Hydroxydopamine-Induced Mitochondrial Damage and Neurotoxicity

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## Abstract

The etiology of Parkinson's disease is not completely understood and is believed to be multifactorial. Neuronal disorders associated to oxidative stress and mitochondrial dysfunction are widely considered major consequences. The aim of this study was to investigate the effect of the synthetic arylidenmalonate derivative 5-(3,4-dihydroxybenzylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (KM-34), in oxidative stress and mitochondrial dysfunction induced by 6-hydroxydopamine (6-OHDA). Pretreatment (2 h) with KM-34 (1 and 10 μM) markedly attenuated 6-OHDA-induced PC12 cell death in a concentration-dependent manner. KM-34 also inhibited $H_2O_2$ generation, mitochondrial swelling, and membrane potential dissipation after 6-OHDA-induced mitochondrial damage. In vivo, KM-34 treatment (1 and 2 mg/Kg) reduced percentage of asymmetry (cylinder test) and increased the vertical exploration (open field) with respect to untreated injured animals; KM-34 also reduced glial fibrillary acidic protein overexpression and increased tyrosine hydroxylase-positive cell number, both in substantia nigra pars compacta. These results demonstrate that KM-34 present biological effects associated to mitoprotection and neuroprotection in vitro, moreover, glial response and neuroprotection in SNpc in vivo. We suggest that KM-34 could be a putative neuroprotective agent for inhibiting the progressive neurodegenerative disease associated to oxidative stress and mitochondrial dysfunction.

**Keywords** KM-34 · Arylidenmalonate derived · Mitochondria · Neuroprotection · Parkinson's disease

## Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder marked by the appearance of Lewy neurites/bodies in the brains of asymptomatic persons and clinical motor symptoms such as bradykinesia, muscular rigidity, resting tremor, and postural instability in symptomatic persons that lost about 80% of striatal or putaminal dopaminergic neurons (Cheng et al. 2010; Hughes et al. 1992; Ross et al. 2004; Sveinbjornsdottir 2016). The motor symptoms appear after years of degenerative process and are preceded by non motor symptoms such as olfactory and mood disturbances (Braak et al. 2004). The mechanisms responsible for the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) remain unknown; however, mitochondrial dysfunction (Exner et al. 2012), oxidative stress (Golembiowska et al. 2013),

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neuroinflammation (Taylor et al. 2013), and iron accumulation in dopaminergic neurons and glial cells in the SNpc of PD patients may contribute to the protein aggregation and dopaminergic neuron death (Hirsch 2009).

The main animal model used to induced dopaminergic degeneration are neurotoxins such as 6-hydroxydopamine (6-OHDA) (Schober 2004), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Jenner 2003), and rotenone (Greenamyre et al. 2010), all have in common mitochondrial dysfunction by inhibiting complexes I, oxidative stress, and neuroinflammation (Blesa and Przedborski 2014).

The inducible parkinsonism in human and monkeys by mitochondrial activity inhibitor is an evidence of the role that mitochondria plays in PD physiopathology (Langston et al. 1983). It is now well understood the role between mitochondrial biology and neurodegeneration in PD, that involves bioenergetic failure, increasing the production of reactive oxygen species (ROS), deregulation of calcium homeostasis, or activation of programmed cell death (Perier and Vila 2012). Remarkably, mitochondria are also implicated in most genetic forms of familial PD: electron transport chain complex IV activity is reduced in an α-synuclein mouse model (Martin et al. 2006); the mitochondrial respiratory capacity is decreased, and oxidative damage is increased in both Parkin-knockout mouse (Palacino et al. 2004) and PTEN-induced putative kinase 1 (PINK1). Changes in expression of PINK1 cause mitochondrial dysfunction, proteasomal deficit, and α-synuclein aggregation in cell culture models of PD (Liu et al. 2009).

6-OHDA induces mitochondria dysfunction by inhibiting complexes I and IV (Glinka and Youdim 1995), and the high affinity to the norepinephrine and dopamine transporters (Redman et al. 2006) explains its accumulation in norepinephrine and dopamine neurons. It has been used as a neurotoxin to generate in vitro and in vivo PD model for developing new therapies during long time and this is still widely used (Kasture et al. 2009; Rauch et al. 2010; Rodriguez-Pallares et al. 2009). The model with unilateral 6-OHDA-lesioning of rats is performed by injecting 6-OHDA directly into SNpc to destroy dopaminergic neurons, or directly into striatum to damage dopaminergic innervations (Ungerstedt 1968).

Based on the central role mitochondria play in neuronal cell death, strategies that target mitochondrial-mediated cell death pathways have particular promise as neuroprotective therapies (Dawson and Dawson 2017). KM-34 (5-(3,4-dihydroxybenzylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione) is a novel synthetic potent antioxidant, with a high capacity of radical sequestration 2,2-Diphenyl-1-picrylhydrazyl (DPPH) ($IC_{50} = 16.26$ μM) and $O_2^{\bullet}$ ($IC_{50} = 11.04$ μM), as well as inhibition of the oxidative processes of deoxyribose and phospholipids. Iron chelation has being proposed as an additional mechanism for free radical sequestration. The displacement of the absorption maxima of the KM-34 indicates the formation of a complex KM-34-$Fe^{2+}$ which reinforces the hypothesis of inhibition of the Fenton-Haber-Weiss reaction for this molecule. The cytoprotective effect of KM-34 on highly oxidizing agents (hydrogen peroxide ($H_2O_2$) and the $Fe^{3+}$/ascorbate system) was also demonstrated in cell cultures (PC12), achieving cellular survival values above 90% (Nuñez-Figueredo et al. 2017). These antioxidant properties of KM-34 demonstrated in vitro, motivate the continuation of the study of this compound as a possible neuroprotective agent in pathologies where iron and free radicals have a toxic role determinant for the development and worsening of the disease, such as PD. The aim of this study was to investigate the effect of KM-34, in different in vitro and in vivo models induced by neurotoxic action of 6-OHDA in view to elucidate a possible protective role and if it is associated to modulation of mitochondrial function.

## Materials and Methods

### Synthesis of 5-(3,4-Dihydroxybenzylidene) -2,2-Dimethyl-1,3-Dioxane-4,6-Dione (KM-34)

A mixture of equimolar amount of 3,4-dihydroxybenzaldehyde 1 (10 mmol) and Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) (Meldrum's acid) 2 (10 mmol) were mixed in a round bottom flask using 30–40 mL of water and was stirred in a round bottom flask using 30–40 mL of water, at room temperature, during 4 h. After the completion of the reaction monitored by thin-layer chromatography (TLC) on silica-gel plates, an amorphous yellow solid was collected by vacuous filtration and gently washed with water or water/ethanol (2:1, V:V) mixture, and appropriately dried to afford 5-(3,4-dihydroxybenzylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione 3 (KM-34). Yield, 81%; mp, 155–156 °C (Fig. 1).

To synthesized KM-34 (Fig. 1), an equimolar amount of 3,4-dihydroxybenzaldehyde (10 mmol) (Fig. 1, molecule 1) and 2,2-dimethyl-1,3-dioxane-4,6-dione (10 mmol Meldrum's acid) (Fig. 1, molecule 2) were stirred in a round bottom flask using 30–40 mL of water, at room temperature during 4 h. After the completion of the reaction (monitored by TLC on silica-gel plates), an amorphous solid (yellow) was collected by vacuous filtration and gently washed with water

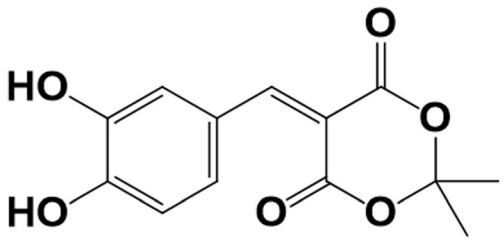



**Fig. 1** Chemical structure of KM-34 (5-(3, 4-dydroxyl-benzylidene)-2, 2-dimethyl-1, 3-dioxane-4, 6-Dione)

or water/ethanol (2:1, V:V) mixture, and appropriately dried. The reaction time was 4 h, yield was 81% and mp was 155–156 °C.

IR (KBr: ν (cm-1): 3503, 3242 (OH) 3109, 2993 (Csp2-H); 1703, 1690 (C = O); 1627(C = C); 1597–1399 (Ar). RMN-1H (DMSO-d6, d6, δ ppm): 8.14 (1H, s, H7); 7.93, (1H, d, Ar, J = 2.13 Hz); 7.54, (1H, dd, J1 = 2.13 Hz, J2 = 8.48 Hz); 6.87 (1d, J = 8.48 Hz); 1.72 (6H, s, H10, H11). RMN-13C (DMSO-d6, δ ppm): 163.5, 160.2 (C4, C6); 157.4 (C7); 153.1, 145.2 (C3′ C4′); 131.2, 123.5, 120.3, 115.6, 109.4 (C5, C1′, C2′, C5′, C6′); 103.8 (C2); 26.8 (C10, C11).

Melting points were determined in open capillary tubes using a Stuart Scientific (UK) apparatus and were no corrected. Both, $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker spectrometer AFP300 (300 MHz-$^{1}H$; 75.2 MHz-$^{13}C$) Bruker AC-250F spectrometer in CDCl3/DMSO-d6. Chemical shifts δ are given in ppm relative to TMS. IR spectra were recorded on KBr disc using a WQF-510-FTIR spectrophotometer apparatus (4000–600 $cm^{-1}$).

## PC12 Cell Culture

PC12 rat pheochromocytoma cell line was purchased from ATCC (#CRL -1721.1 PC-12 ADH, *Rattus norvegicus*, Manassas, VA, USA). These cells were cultured in medium containing Roswell Park Memorial Institute (RPMI-1640) (Cultilab, SP, Brazil), supplemented with L-glutamine (Cultilab, SP, Brazil), 10% inactivated fetal bovine serum (FBS) (Cultilab, SP, Brazil) and 5% inactivated horse serum (Cultilab, SP, Brazil), and 1% penicillin and 1% streptomycin (Cultilab, SP, Brazil). PC12 cells were cultured until confluence in 10-mm polystyrene plates (TPP, Trasadingen, Switzerland), trypsinized and replated on 96-well ($7.5 \times 10^3$ cells/$cm^2$) polystyrene culture plates dishes (TPP, Trasadingen, Switzerland), and maintained in an incubator with humidified atmosphere with 5% of $CO_2$ and 37 °C.

## Cell Treatment and MTT Assay

MTT is a water soluble tetrazolium salt that is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria in healthy cells (Mosmann 1983; Shearman et al. 1995).

Two hundred millimmolar (200 mM) stock solutions of KM-34 were prepared in dimethylsulfoxide (DMSO). The final concentration was prepared by dilution in RPMI-1640 medium without FBS. The cells were grown on 96-well plate at 37 °C for 24 h. After discarding the old medium, the cells were exposed to 6-OHDA (50 to 400 μM) or treated with KM-34 (0.001 to 100 μM) for 24 h or pretreated for 2 h with KM-34 (1 and 10 μM) followed by co-treatment with 100 μM 6-OHDA for 24 h, and mitochondrial dehydrogenases activity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) assay.

The negative control group was treated with DMSO diluted in the culture medium or reaction medium at the higher equivalent volume used in the treated groups (0.05%) and showed no significant effect on analyzed parameters as compared to cells that did not receive diluents.

The mitochondrial dehydrogenases activity was quantified using the conversion of yellow MTT by living cells to purple MTT formazan as an index (Berridge and Tan 1993). Control and treated cells were incubated with MTT at a final concentration of 1 mg/mL for 2 h. Thereafter, cells were lysed with 20% (*w*/*v*) sodium dodecyl sulfate (SDS) and 50% (*v*/v) dimethylformamide (DMF) (pH 4.7). Plates were incubated overnight at 37 °C to dissolve formazan crystals. The optical density of each sample was measured at 492 nm using a Varioskan™ Flash Multimode Reader spectrophotometer (Thermo Plate, Brazil). Three independent experiments were carried out with eight replicate wells for each analysis. Results from the MTT test were expressed as percentages of the 492 nm absorbance of the treated groups as compared to the control groups.

## Experimental Animals

Male Wistar rats (230–250 g) were obtained from Center for the Production of Laboratory Animals (CENPALAB), Mayabeque, Cuba and for acclimation, housed in the animal care facility for 1 week prior to in vivo experiments. Rats were housed in a temperature-controlled environment (22 ± 2 °C) with a 12 h light/dark cycle and had access to food, with standard diet for rodents (CMO 1000, CENPALAB) and water ad libitum. Animal housing, care, and the application of experimental procedures were in accordance with institutional guidelines and were conducted according to approved protocols (Animal Care Committee from CIDEM, Havana, Cuba) and in accordance with the Declaration of Helsinki (1964).

## 6-OHDA-Induced Parkinson Model

### Surgical Procedure

Rats were anesthetized by means of intraperitoneal (i.p.) injection of chloral hydrate (420 mg/kg weight, Merck (Darmstadt, Germany) and placed in a stereotactic frame for rodent surgery (Stoelting Instruments, EE.UU).To achieve lesioning of the nigroestriatal pathway, 3 μL of 6-OHDA (8 μg/3 μl in 0.01% ascorbic acid dissolved in saline preventing heat and light exposure) was stereotactically injected into the right SNpc (anteroposterior (AP): − 4.4, mediolateral (ML):+ 1.2, dorsoventral (DV):− 7.8) using a

10 μl Hamilton Syringe (Sigma, St Louis, MO, USA) with 28-gaugeneedle. Syringes were lowered into the brain at a rate of 2 mm/min. 6-OHDA was injected at a rate of 1 μl/min, and the syringe was left in place for 5 min after injection before being drawn back at a rate of 2 mm/min. Rats were monitored for 2 h post surgery before returning to the animal housing facility. An artificial tears (Julio Trigo, Havana, Cuba) was applied to protect against corneal drying during all surgical procedure and until the animal recovered from anesthesia. One separate group of control rats received saline (6-OHDA vehicle) injections following the same procedures (Pavon-Fuentes et al. 2004). Taking as reference, the Bregma point determined the right SNpc coordinates (mm) (Büttner-Ennever 1997).

## Experimental Groups

Vehicle (0.05% carboxymethylcellulose (CMC) solution) and KM-34 were administered by oral gastric (i.g) gavage 30 min before stereotaxic surgery, daily for 7 days. The variability in the dosing volumes was mitigated by adjusting the concentration to ensure a constant volume (10 ml/kg). Immediately before use, KM-34 was suspended in CMC solution and administered i.g with a gavage as a single dose (0.5, 1, or 2 mg/Kg). Animals were randomly divided into five experimental groups: (1) rats with lesion in the SNpc induced by 6-OHDA and treated with CMC (6-OHDA), (2) rats with lesion in the SNpc induced by 6-OHDA and treated with KM-34 (0.5 mg/kg), (3) rats with lesion in the SNpc induced by 6-OHDA and treated with KM-34 (1 mg/kg), (4) rats with lesion in the SNpc induced by 6-OHDA and treated with KM-34 (2 mg/kg), and (5) rats with false lesion (6-OHDA vehicle injected in the SNpc right) (vehicle) and treated with CMC.

## Behavioral Studies

The study of sensorimotor dysfunction associated with neurotoxic injury of SNpc was performed using two behavioral tests that assess the spontaneous activity of the animals: open field test (BureŠ et al. 1976; Schallert et al. 2000) and the cylinder test (Allbutt and Henderson 2007). Seven days after SNpc lesion, both behavioral tests were performed. Behavioral studies were conducted under appropriate conditions of silence and lighting.

## Cylinder Test

Seven days following surgery, the animals were placed individually inside a transparent cylinder (21 cm in diameter and 34 cm high). The cylindrical shape encouraged rearing and vertical exploration of the walls with the forelimbs (Schallert et al. 2000). No habituation to the cylinder prior to the experiments was allowed. A total of 20 forepaw touches (right, left and both) were counted (Decressac et al. 2012; Nunez-Figueredo et al. 2016). Rats were put in a glass cylinder and the percentage asymmetry was determined. (Chan et al. 2010; Nunez-Figueredo et al. 2016).

## Open Field Test

Seven days following surgery, rats were placed in a clean open field (UGO BASILE, Multiple Activity Cage, Italia) container (45 × 45 cm) surrounded by plexiglass walls in a quiet, well-lit room. The cubicle rests on a sturdy black steel base, fitted with four vertical steel bars with notches so the vertical detection system is correctly fixed. Sensors are infrared light emission systems capable of recording the movements of animals. Motor and exploratory activity was video recorded and tracked for 10 min. Total number of ambulatory episodes were recorded using a tracking software (UGO BASILE, Data Acquisition Software, CUB 2005), monitored on a computer. The container was sanitized with 70% ethanol between each trial and allowed to air dry (BureŠ et al. 1976; De Jesus-Cortes et al. 2015).

## Immunohistochemical Procedures

After behavioral studies, animals were deeply anesthetized with ketamine (50 mg/kg i.p) and Xylazine (50 mg/kg i.p). Rats were perfused transcardially, and brains were post-fixed in 4% paraformaldehyde (PFA; Sigma, St. Louis, MO) for 48 h at 4 °C in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. After extraction, perfused and post-fixed brains were placed into a 30% sucrose solution in saline until they sank. Coronal plane cryostat-cut sections (25-μm-thick) spanning the entire midbrain, and SNpc were processed for Immunohistochemistry detection of tyrosine hydroxylase (TH+) and glial fibrilary acidic protein (GFAP) (Marti et al. 2007). For immunofluorescence, tissue sections containing the SNpc which were incubated were treated with 0.5% citrate buffer (65 °C, with constant shaking) for 30 min to maximize antibody penetration into tissue. Non-specific Fc binding sites were blocked with 10% horse serum. Tissue sections were incubated at 4 °C with primary antibodies overnight, diluted with PBS containing 1% normal horse serum, 0.5% Triton X-100 (Cultilab, SP, Brazil), and sodium azide (Cultilab, SP, Brazil) 0.01%. After, tissues were incubated with TH+ antibody solution (polyclonal rabbit anti-TH antibody; 1:1000; Abcam) to identify dopaminergic neurons or GFAP antibody solution (polyclonal rabbit anti-GFAP antibody 1:300; DAKO) to identify astrocytes. The sections were incubated for 4 h in labeled appropriate secondary antibody Alexa 594 (1:1000, Abcam). After PBS washes, the sections were incubated with DAPI solution 4,6-Diaminodinoz-phenylondole (DAPI, Invitrogen Cat.D13064, 1:1000) in 1X PBS for 30 min. Thereafter, they were analyzed by fluorescent microscopy and photographed (Olympus AX70). The number of

TH+ and GFAP cells in the SNpc was quantified manually using NIH (National Institutes of Health) ImageJ software 1.47v (Bethesda, MD, USA) by an investigator blinded to treatment regimen. Three coronal photomicrographs through the SNpc (right hemispheres) was analyzed (AP distance from bregma (mm): − 0.44 (AP), + 0.12 (ML), and − 0.78 (DV)) by each animal. Quantification of the percentage of TH positive neurons was performed comparing the ipsilateral SNpc with the contralateral control SNpc as previously described (Duty and Jenner 2011; Kim et al. 2016).

## Mitochondrial Protection Assay

### Isolation of Rat Brain Mitochondria

Mitochondria were isolated from male Wistar rats of approximately 230 g as described by (Mirandola et al. 2010), with minor modifications. Briefly, rats were sacrificed by decapitation, and their brains were rapidly removed (within 1 min) and placed into 10 ml of ice-cold "isolation buffer" containing 225 mM mannitol, 75 mM sucrose, 1 mM K-EGTA, 0.1% bovine serum albumin (BSA, fatty-acid free), and10 mM K-HEPES (pH 7.2). The cerebellum and underlying structures were removed, and the remaining tissue, which was considered the forebrain, was used to isolate brain mitochondria. The forebrains were cut into small pieces using surgical scissors and extensively washed in isolation buffer. The tissue was then manually homogenized in a glass Potter-Elvehjem homogenizer with both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000*g* in a Hettich Zentrifuge (Germany), Rotina 380R. After centrifugation, the supernatant was centrifuged for 8 min at 12,000*g*. The pellet was resuspended in 10 ml isolation buffer containing 20 ml of 10% digitonin (Sigma, St. Louis, MO), which was used to release synaptosomal mitochondria, and recentrifuged for 10 min at 12,000*g*. The supernatant and the upper light layer of the pellet was discarded, and the dark pellet was resuspended in isolation buffer devoid of EGTA. This homogenate was then centrifuged for 10 min at 12,000*g*. The supernatant was discarded, and the final pellet gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 30–40 mg/mL. The entire procedure was carried out at 4 °C. The respiratory control ratio (state 3/state 4 respiratory rate) was greater than 4, measured using 5 mM succinate as a substrate.

## Continuous-Monitoring Mitochondrial Assays

Mitochondrial membrane potential (Ψm) was determined spectrofluorimetrically using 10 mM safranine O (Sigma, St. Louis, MO) as a probe (Pardo-Andreu et al. 2011) in a Varioskan™ Flash Multimode Reader (Thermo Plate) at 495/586 nm excitation/emission wave lengths; these assays were performed in the presence of 0.1 mM EGTA and 2 mM $K_2HPO_4$. Mitochondrial swelling was estimated spectrophotometrically from the decrease in apparent absorbance at 540 nm using a Varioskan™ Flash Multimode Reader (Thermo Plate). ROS were monitored spectrofluorimetrically using 1 μM Amplex red (Molecular Probes, OR, USA) and 1 UI/mL horseradish peroxidase (Sigma, St. Louis, MO) at 563/587 nm excitation/emission wavelengths (Pardo-Andreu et al. 2011). For all assays, mitochondria were energized with 5 mM potassium succinate (plus 2.5 mM rotenone) in a standard medium consisting of 125 mM sucrose, 65 mM KCl, and10 mM HEPES-KOH, pH 7.4, at 30 °C. The mitochondrial impairment was induced by 6-OHDA 1 mM, and the mitochondrial parameters were determinate in the absence or presence of KM-34 (1 and 10 μM). Relative fluorescence or absorbance measurements were recorded for 600 s.

## Statistical Analysis

Results are expressed as means ± SEM. One-way ANOVA followed by the Student-Newmann–Keuls test was used to determine the statistical differences among groups differing in only one parameter. All analyses were performed with three independent experiments carried out with eight replicate wells. Values of $p < 0.05$ were considered to be significant.

Normal distribution and homogeneity of variance of the data were tested by the Kolmogorov–Smirnov and Levene tests, respectively. Significant differences between groups were determined by one-way ANOVA, followed by Tukey's post hoc analysis. In all cases, the statistic program using was GraphPad Instat, Version 5, for Windows© (San Diego, CA, USA, www.graphpad.com). All data, at the 95% confidence interval, are expressed as the means ± standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$. All analysis was conducted by one experimenter who was blinded to the experimental group designations.

# Results

## Synthesis of 5-(3,4-Dihydroxybenzylidene) -2,2-Dimethyl-1,3-Dioxane-4,6-Dione (KM-34)

Arylidenmalonates had been thoroughly prepared starting from aromatic aldehydes and 1,3-dicarbonylic compounds by means of Knoevenagel condensation reaction. Although condensation reaction, which evolved with the extrude of a water molecule, are usually conducted in organic dry media; the preparation of compound KM-34 was conducted and reported here, using aqua media without catalyst, which brings the advantage of favoring the precipitation of this particular

product during the reaction, also standing for a green (eco-friendly) procedure in synthesis. IR spectra show hydroxyl groups bands at 3503 and 3242 $cm^{-1}$, and the intense characteristic carbonylic band at 1701 and 1690 $cm^{-1}$. $^{1}H$–NMR shows four aromatic protons between 7.93–6.87 ppm, while the olefin proton H-7 appears as a singlet at 8.14 ppm. Finally, a singlet at 1.72 ppm which integrates six protons, corresponds to the methyl groups. $^{13}C$ NMR spectra confirmed the proposed structure.

## KM-34 Attenuates Deleterious Effects Induced by 6-OHDA in PC12 Cells

MTT test, which measures the reduction of the tetrazolium salt (MTT) to the purple formazan by mithocondrial dehydrogenase enzymes in living cells demonstrated that 24 h exposure to 6-OHDA induced a dose-dependent reduction of dehydrogenase activity in PC12 cell culture. The 6-OHDA significant cytotoxicity was first visualized at concentration of 100 μM (60.5% ± 5%) (Fig. 2a). On the other hand, KM-34 (0.001–100 μM) did not reduce dehydrogenase activity in PC12 cell culture (Fig. 2b). Moreover, it demonstrated that 24 h exposure to 6-OHDA (100 μM) reduced dehydrogenase activity for 66.5% ± 3% (Fig. 2c). 6-OHDA exposed PC12 cells that was pretreated for 2 h with 1 and 10 μM KM-34 presented dehydrogenase activity reduction of 86.6 ± 4 and 91.57 ± 3, respectively, which demonstrated that KM-34 attenuates deleterious effects induced by 6-OHDA (Fig. 2b).

## KM-34 Protects the Mitochondria from 6-OHDA-Mediated Mitochondrial Swelling, ROS Production, and Membrane Potential Dissipation

The direct relation between mitochondrial dysfunction and PD came from the *post-mortem* description of complex I deficiency in the substantia nigra of patients with PD (Zsurka and Kunz 2013). The neuronal cell death induced by (6-OHDA has been suggested to be mediated by mitochondrial permeability transition pores (mPTP), the collapse of mitochondrial membrane potential (Cassarino et al. 1999; Lotharius et al. 1999), inhibition of mitochondrial complexes I and IV (Glinka and Youdim 1995), and elevated ROS production (Tanaka et al. 2006).

In the next set of experiments, we assessed the effects of KM-34 on 6-OHDA-induced mPTP opening, as estimated by the swelling of succinate energized rat brain mitochondria and monitored as mitochondrial swelling assessed by Δabsorbance increase. Figure 3a shows that 100 μM 6-OHDA induced mitochondrial swelling, as revealed by the increase in Δabsorbance of the mitochondrial suspension at 540 nm (column 3: 6-OHDA without KM-34). This swelling was associated with a faster mitochondrial membrane potential dissipation (column 3: 6-OHDA without KM-34) (Fig.3b)

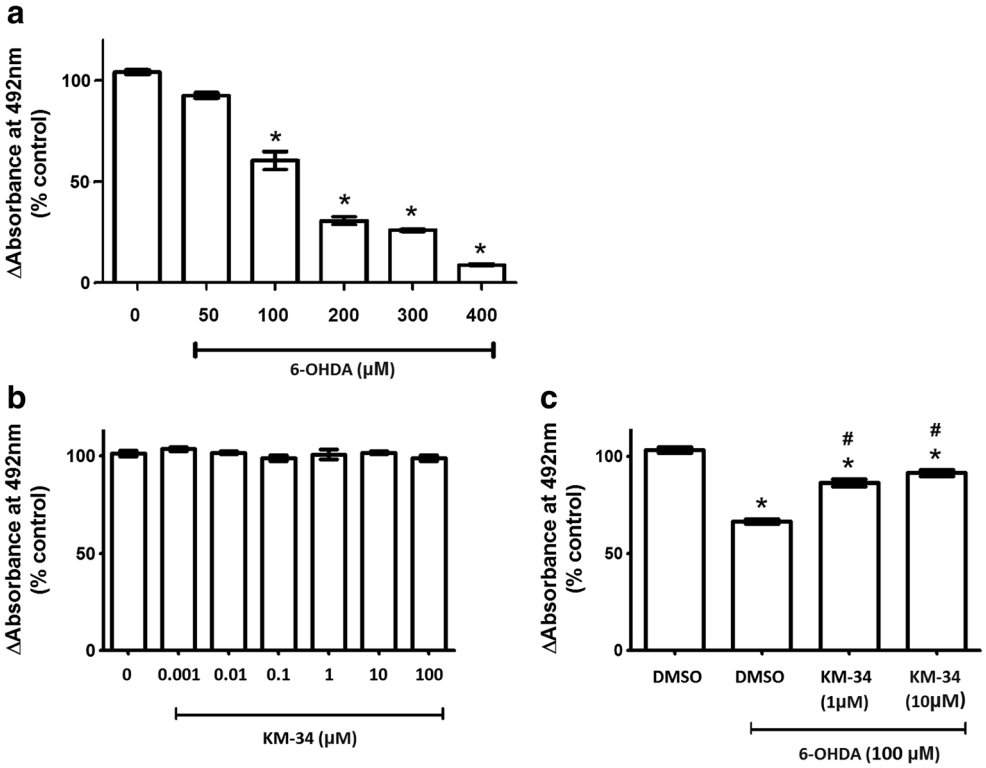
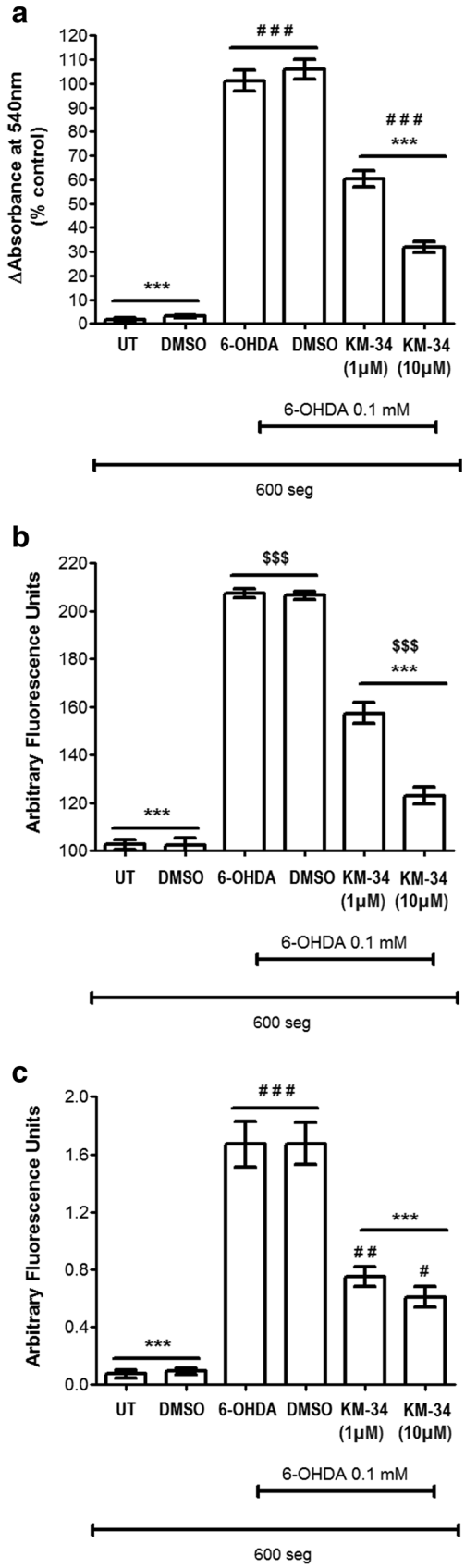



**Fig. 2** Protective effects of KM-34 on succinate dehydrogenase activity in PC12 cells by MTT test. **a** Succinate dehydrogenase activity expressed by percentage of absorbance at 492 nm in PC12 cells exposed to 6-OHDA (50–400 μM) for 24 h or under control (without 6-OHDA). **b** Succinate dehydrogenase activity expressed by percentage of absorbance at 492 nm in PC12 cells treated with KM-34 (0.0001–100 μM) for 24 h or under control conduction (0.05% DMSO). **c** Succinate dehydrogenase activity in PC12 cells pre-treated for 2 h with KM-34 and/or co-treated with KM-34 more 6-OHDA for 24 h. The data are presented as the means ± S.E.M. of three independent experiments. *$p < 0.0001$, compared to control group and #$p < 0.0001$ compared to 6-OHDA group by one way ANOVA analysis, followed by Tukey's multiple comparison post hoc test




◀ **Fig. 3** Mitoprotective effects of KM-34 against **a** 6-OHDA-mediated mitochondrial swelling, **b** membrane potential dissipation, and **c** $H_2O_2$ generation. Rat brain mitochondria (0.5 mg/mL) were incubated at 30 °C under continuous stirring in standard medium that had been supplemented with 0.1 mM 6-OHDA, as described in Section 2. Relative fluorescence or absorbance measurements were recorded for 600 s. For the estimation of the mitochondrial membrane potential (**b**), the medium also contained 5 μM Safranine O, and for ROS production, the medium also contained Amplex Red 1 μM and 1 UI/ml horse radish peroxidase (**c**). The data are presented as the means ± S.E.M. of three experiments that were conducted using different mitochondrial preparations. $^{***}p < 0.001$, compared to 6-OHDA group and $^{###}p < 0.001$, $^{##}p < 0.01$, and $^{#}p < 0.05$ compared to UT group by one way ANOVA analysis, followed by Tukey's multiple comparison post hoc test were performed

and a significant increase in ROS levels (Fig.3c), all induced by 0.1 mM 6-OHDA. No mitochondrial swelling presence, membrane potential dissipation, and a significant increase in ROS levels, in rat brain mitochondria without 6-OHDA untreated (UT) and DMSO (KM-34 vehicle). KM-34 at both concentrations (1–10 μM) completely inhibited swelling ($p < 0.001$), mitochondrial membrane potential dissipation ($p < 0.001$), and ROS production ($p < 0.001$) in rat brain mitochondria treated with 100 μM 6-OHDA.

## KM-34 Preserves Motor Function in the 6-OHDA Lesioned Rat

The cylinder test was used as a tool for assessing fine motor activity, through the percentage of times the animal presents vertical exploratory activity, touches the cylinder wall with the front legs, ipsilateral to a lesion, contralateral to a lesion, and with both legs simultaneously. The preferential use of one extremity, in hemiparkinsonian rats, is expressed like percentage (%) asymmetry. The 6-OHDA injured rats (6-OHDA, second column, Fig. 4a) that did not receive any treatment, is presenting a high percentage of asymmetry, close to 90% (Fig. 4a), compared to the control group (Veh), 6% ($p < 0.001$). Damaged animals with 6-OHDA and pre-treated 30 min before surgery with KM-34 (2 and 1 mg/Kg), presented a significantly lower percentage of asymmetry ($p < 0.001$) when compared to injured animals without treatment, when evaluated in cylinder test, 10 and 17%, respectively.

In the assessment of vertical exploratory activity (rearing), evaluated in the open field test, the results showed that the 6-OHDA lesioned group without further treatment showed a decrease in the number (52 rearings) of rearing when compared to the control (Veh) group (214 rearings) ($p < 0.001$). In the 6-OHDA-lesioned groups treated with KM-34, group 2 mg/Kg (174 rearings) and 1 mg/Kg (144 rearings), showed an increase in the number of rearing as compared to 6-OHDAlesioned group, ($p < 0.01$) and ($p < 0.05$), respectively (Fig. 4b).

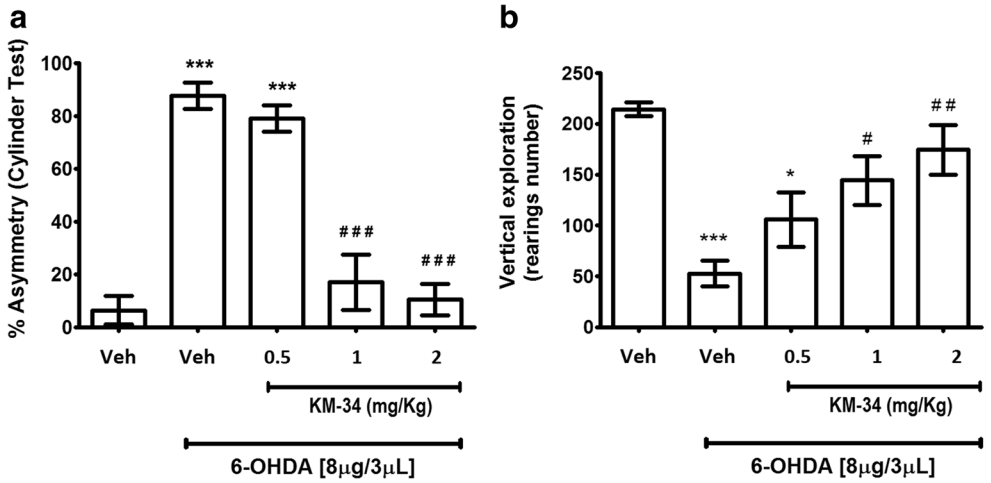



**Fig. 4** Neuroprotective effects of KM-34 on damage induced by 6-OHDA in rats after 7 days of treatment. Animals were placed individually in a transparent, perspex cylinder (21 cm diameter × 34 cm height) and a total of 20 forepaw touches were counted. **a** Reduced % asymmetry, animals treated with KM-34 (2 and 1 mg/Kg) showed decreased of motor damage with respect to untreated 6-OHDA exposed animals. **b**The animals were gently placed in the center position of the open field and allowed to freely explore the area for 10 min. Open field test showed a significant decrease in the total vertical exploration of 6-OHDA-exposed animals compared to Vehicle (Veh) animals ($p < 0.001$) and also with 6-OHDA-exposed animals that were treated with KM-34 (2 mg/Kg) ($p < 0.01$) and (1 mg/Kg) ($p < 0.05$). Experimental groups (Cylinder test): Vehicle rats ($n = 10$), 6-OHDA lesion ($n = 9$), 6-OHDA lesion and KM-34 (2 mg/Kg) ($n = 10$), 6-OHDA lesion and KM-34 (1 mg/Kg) ($n = 7$), 6-OHDA lesion and KM-34 (0.5 mg/Kg) ($n = 6$). (Open field test): $n = 5$ for all groups. The data are presented as the means ± S.E.M. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared to 6-OHDA-lesioned group, by one way ANOVA analysis, followed by Tukey's multiple comparison post hoc test were performed

### KM-34 Inhibits Dopaminergic Neuron's Degeneration and Reactive Astroglioses Induced by 6-OHDA

Immunohistochemistry assay for TH showed a significant loss of TH-positive neurons at 7 days in the right SN of 6-OHDA injected rats compared to ipsilateral (right) SNpc of vehicle-injected rats (29.9 ± 4.2%, $^{*}p < 0.001$) (Fig. 5b, d). When neuronal degeneration was quantified and expressed as the percentage of TH-positive neurons on the ipsilateral SNpc compared with TH-positive neurons on the contralateral control SNpc, there is no evidence of protective effect of 0.5 mg/kg KM-34 treatment against 6-OHDA-induced DA neuron's degeneration in the right SNpc of rats (41.5 ± 7.1% $TH^+$ neurons, $^{*}p < 0.001$) (Fig. 5d). Intermediate (1 mg/mL) and higher dose (2 mg/mL) of KM-34 demonstrated high effective inhibition of 6-OHDA-induced degeneration, which was evidenced by 88.2 ± 5.9 and 99.1 ± 6.3% $TH^+$ neurons on the right SNpc ($^{###}p < 0.0001$) (Fig. 5d).

There are evidences that astrocyte responses to dopaminergic denervations by stereotaxic injection of 6-OHDA by an increased amount of GFAP-immunoreactivity detectable after 24 h and remained after 1 month (Stromberg et al. 1986). It was observed a significant increase in the number of GFAP-positive cells by immunohistochemistry assay (75.5 ± 12.8%, $^{**}p < 0.001$) at 7 days in the right SN of 6-OHDA-injected rats compared to ipsilateral (right) SN of vehicle-injected rats (45.3 ± 8.3%) (Fig. 6 b, f). A lower number of GFAP-positive cells was evidenced at 7 days in the ipsilateral SN of rats treated with 1 mg/mL KM-34 (44.8 ± 18.9%, $^{\#}p < 0.001$) and 2 mg/mL KM-34 (51.1 ± 12.1%, $^{\#}p < 0.001$) when compared to rats only injected with 6-OHDA (Fig. 6d–f).

## Discussion

Mitochondrial dysfunction is implicated in normal as well as pathological aging, especially in many neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, amiotrophic lateral sclerosis, and PD (Johri and Beal 2012). The searches for new molecules that present mitoprotective effect have been growing in scientific community (Murphy 2008; Nunez-Figueredo et al. 2014).

Evidence suggests that reduction of MTT can also be mediated by nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) within the cells and out of mitochondria (Berridge and Tan 1993). In this work, we demonstrated that a new arylidenmalonate derivative compound reduces mitochondrial dysfunction induced by 6-OHDA in PC12 cells by MTT test and confirmed the KM-34 mitoprotective effect on rat isolated mitochondria in view of mitochondrial potential dissipation, swelling, and ROS production.

Mitochondrial health is essential for neuronal functions, which includes axonal transport, production of synaptic vesicles, preservation of ion gradients, and membrane potential. In this sense, neurons have a high demand for adenosine triphosphate (ATP) which depend predominately on oxidative phosphorylation, and there is clear association between

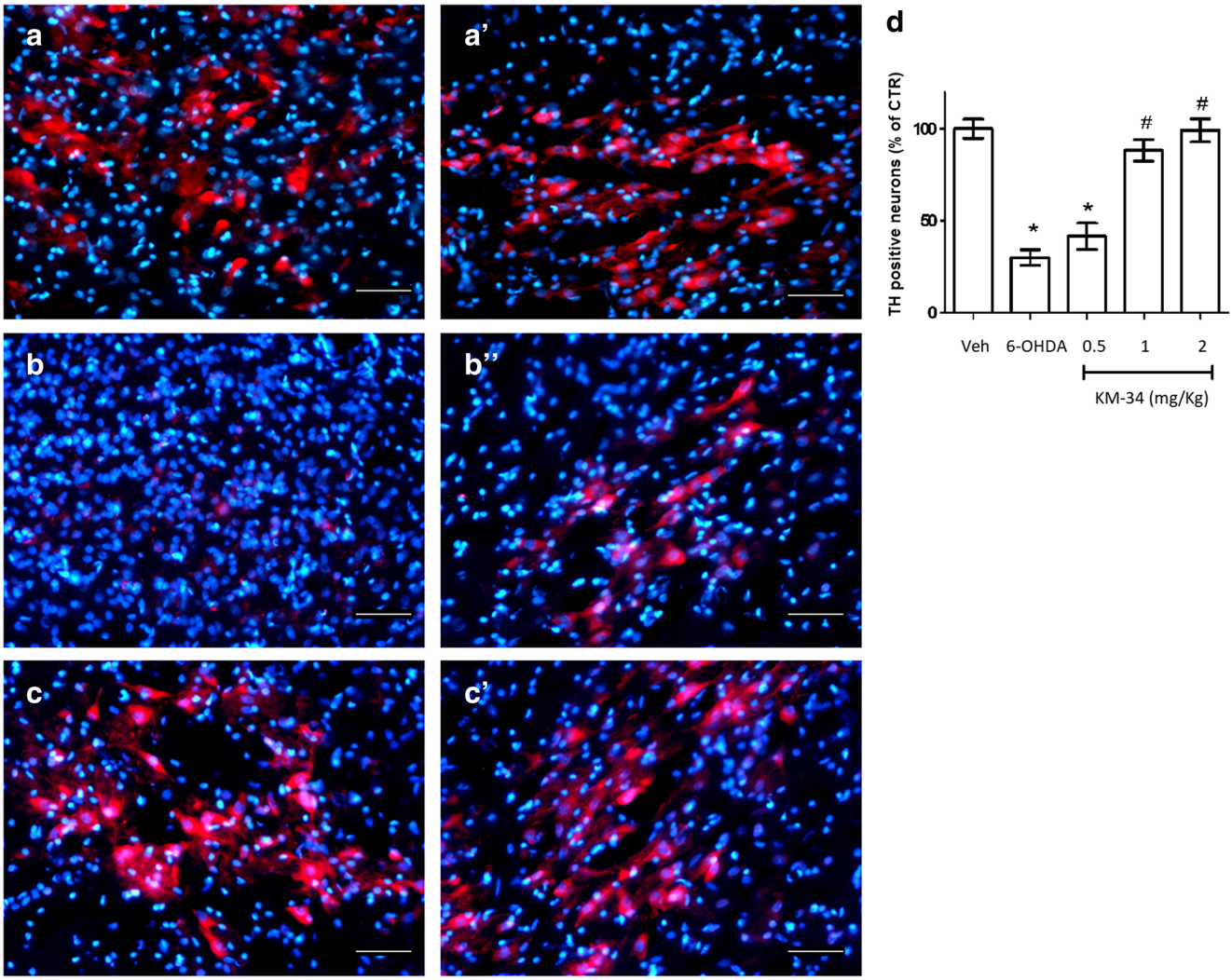



**Fig. 5** Neuroprotective effects of KM-34 on damage induced by 6-OHDA in rats after 7 days of treatment. **a** Immunohistochemistry to TH (red) and DAPI-staining chromatin (blue) in right (ipsilateral) SN of rats under control conditions (untreated 6-OHDA exposed animals). **a**' Immunohistochemistry to TH+ and DAPI-staining chromatin in left (contralateral) SN of rats under control conditions (untreated 6-OHDA-exposed animals). **b**, **b**' Immunohistochemistry to TH+ and DAPI-staining chromatin in right and left (respectively) SN of rats exposed to 6-OHDA no treated with KM-34. **c**, **c**' Immunohistochemistry to TH and DAPI staining chromatin in right and left (respectively) SN of rats exposed to 6-OHDA treated with KM-34 (2 mg/Kg). Obj. 20 × 0.70, scale bars = 50 μm. d Fluorescent cells quantified and plotted as the percentage of TH-positive neurons on the ipsilateral SNpc compared with TH-positive neurons on the contralateral control SNpc. The values are the mean ± S.E.M ($n = 3$) $^{*}p < 0.05$, $^{**}p < 0.001$ compared to Veh group, $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ compared to 6-OHDA group. The statistical significance was assessed by using one way ANOVA analysis, followed by Tukey's multiple comparison post hoc test

mitochondrial dysfunction and nervous system disorders (Rugarli and Langer 2012). The complexes I and IV inhibitor, 6-OHDA, have been used to develop dopaminergic degeneration and behavioral changes in rodents, that is associated with PD pathogenesis (Glinka and Youdim 1995). In this study, we demonstrated a 6-OHDA-induced motor deficits associated to reduction in percentage of $TH^+$ cells in SNpc of rats, which were inhibited by treatment with KM-34. Mitoprotective agents may present different kinds of mechanisms action, for example, associated to antioxidative action (Nunez-Figueredo et al. 2014), inducible of mitophagy action (East and Campanella 2016; Hou et al. 2015), or promotion of mitochondrial biogenesis (Hasegawa et al. 2016). Based on the antioxidant properties of KM-34 demonstrated by Nunez-Figueredo et al. (2017), such as inhibition of iron-induced brain lipid peroxidation, inhibition of 2-deoxyribose degradation, inhibition of superoxide radicals generation, and inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical reduction, we consider the neuroprotetor and mitoprotector effects of KM-34 against 6-OHDA associated to a direct reaction with free radicals and chelating the metal ions responsible for the production of ROS.

It is known that astrocytes respond to dopaminergic denervations induced by 6-OHDA (Stromberg et al. 1986). Activation of astrocytes results on phenomenon named astrogliosis associated with changes in the expression of many

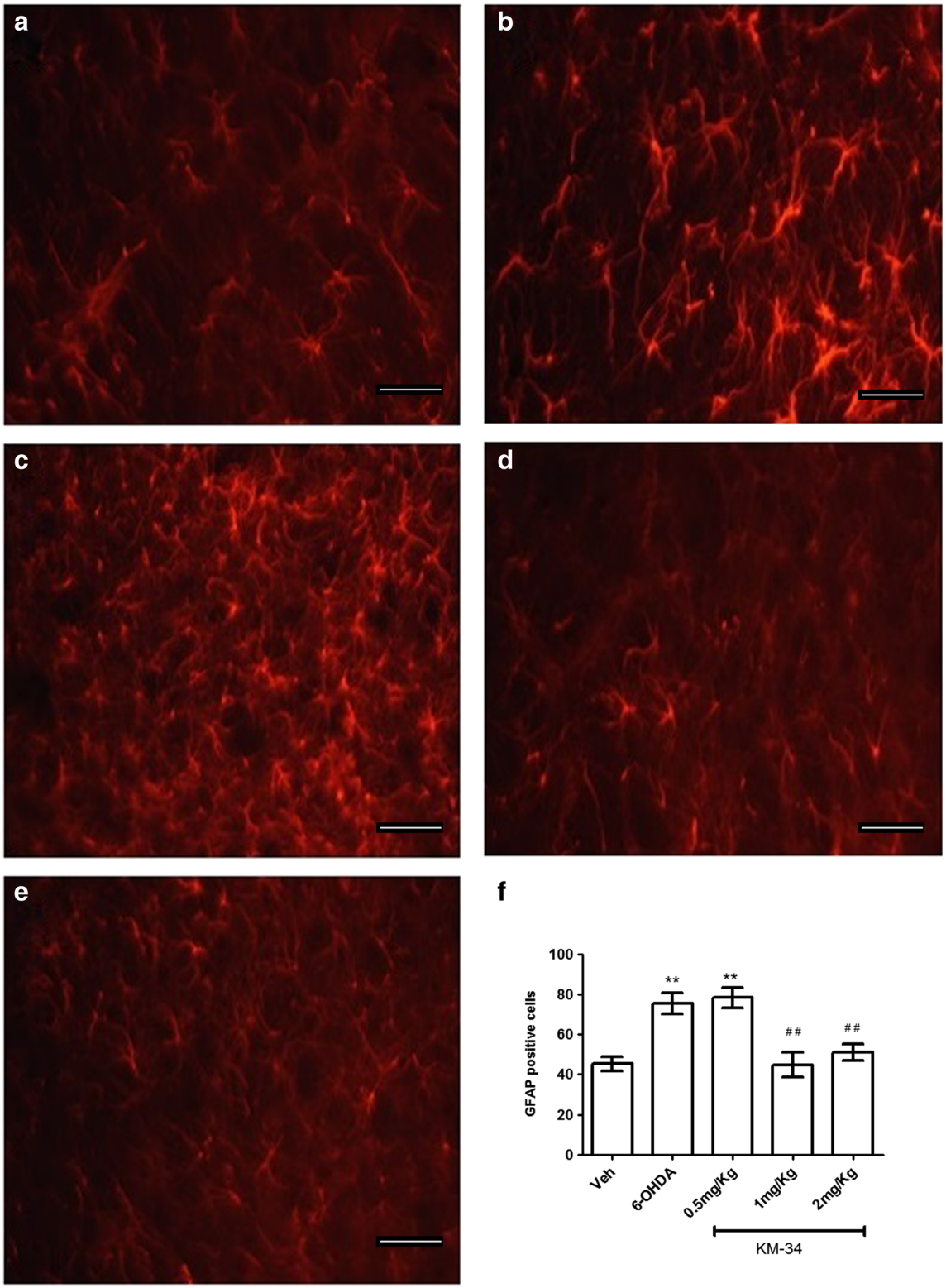



**Fig. 6** Protective effects of KM-34 on damage induced by 6-OHDA in rats after 7 days of treatment. a Immunohistochemistry to GFAP in right (ipsilateral) SNpc of rats under control conditions (untreated 6-OHDA-exposed animals). b Immunohistochemistry to GFAP in right SNpc of rats exposed to 6-OHDA no treated with KM-34. **c**–**e** Immunohistochemistry to GFAP in right SN of rats exposed to 6-OHDA treated with KM-34 (0.5, 1, and 2 mg/Kg, respectively). Obj. 20 × 0.70, scale bars = 50 μm. f The number of GFAP-positive cells was quantified and plotted. The values are the mean ± S.E.M ($n = 3$) $^{**}p < 0.001$ compared to Veh group, $^{\#\#}p < 0.001$ compared to 6-OHDA group. The statistical significance was assessed by using one way ANOVA analysis, followed by Tukey's multiple comparison post hoc test

genes, as GFAP, and characteristic morphological hallmarks, and has beneficial or harmful influence in CNS disorders such as neurodegenerative diseases (Pekny et al. 2014). In this work, it was demonstrated an increase of GFAP-positive cells on lesioned SNpc side in rats, that was inhibited to KM-34 treatment. We suggest that preventive effect of KM-34 against neuronal degeneration eliminated the stimulus to glial response and neuroinflammation (Glass et al. 2010).

## Conclusion

We concluded that KM-34 presents mitoprotective and neuroprotective properties and suggest it could be a putative neuroprotective agent for it inhibits the progressive neurodegeneration associated to Parkinson disease.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no competing interests.

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