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Effects of cinnamon oil and its main constituents, cinnamic acid and cinnamaldehyde, on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration in PC-12 cells

[Efectos del aceite de canela y sus principales componentes, ácido cinámico y cinamaldehído en la neurodegeneración inducida por 1-metil-4-fenil-1,2,3,6-tetrahidropiridina en células PC 12]

Roodabeh Bahramsoltani[1,2], Zahra Shahpiri[1,2], Mohammad Hosein Farzaei[3], Leila Hosseinzadeh[3], Davood Rezazadeh[4], Morteza Pourfarzam[5] & Roja Rahimi[1,2]

[1]Department of Traditional Pharmacy, School of Persian Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2]PhytoPharmacology Interest Group (PPIG), Universal Scientific Education and Research Network (USERN), Tehran, Iran
[3]Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4]Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[5]Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Reviewed by:
Jianzhou Tong
Baoding First Central Hospital
China

Miguel Avila
Universidad de Las Américas
Chile

Correspondence:
Roja **RAHIMI**
rojarahimi@gmail.com

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Abstract: Cinnamon (*Cinnamomum verum* J. Presl) is a well-known medicinal plant considered as an effective treatment for neurological disorders based on Persian medicine. The aim of the present study was assessing the effect of cinnamon oil, cinnamic acid, and cinnamaldehyde, on the *in vitro* model of Parkinson's disease (PD). Cinnamon oil, prepared in sesame oil, was phytochemically analyzed using high performance liquid chromatography (HPLC). Pheochromocytoma-12 (PC-12) cells were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as an *in vitro* model of neurodegeneration in PD. Cell viability, activity of caspase enzymes, and formation of reactive oxygen species (ROS) were evaluated. MPTP significantly decreased cell viability and increased Casp activity, as well as ROS formation. Cinnamon oil and cinnamic acid at 200 µg/mL could significantly reverse MPTP-induced abnormalities in PC-12 cells including Casp activity and ROS formation. Our study supports the beneficial effect of cinnamon oil in neurodegeneration. Further investigations are needed to clarify the mechanisms and main active components.

Keywords: Persian medicine; Parkinson; Neurodegeneration; Cinnamon; Cinamic acid; Cinamaldehyde

Resumen: La canela (*Cinnamomum verum* J. Presl) es una planta medicinal muy conocida, y considerada como un tratamiento efectivo para patologías neurológicas según la medicina persa. El objetivo de este estudio fue evaluar el efecto del aceite de canela, el ácido cinámico, y el cinamaldehído, en un modelo in vitro de la enfermedad de Parkinson (PD). El aceite de canela, preparado en aceite de sésamo, fue analizado fitoquímicamente usando cromatografía líquida de alta eficacia (HPLC). Se trataron células con feocromocitoma-12 (P-12) usando 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) como un modelo in vitro de neurodegeneración en PD. Se evaluó la viabilidad celular, actividad de enzimas caspasa, y formación de especies reactivas del oxígeno (ROS). El tratamiento con MPTP disminuyó significativamente la viabilidad celular y aumentó la actividad casp, así la formación de ROS. Aceite de canela y ácido cinámico a 200 µg/mL podría revertir significativamente las anormalidades inducidas por MPTP en células PC-12, incluyendo la actividad casp y la formación de ROS. Nuestro estudio entrega sustento sobre los efectos benéficos del aceite de canela en la neurodegeneración. Se requiere más investigación para clarificar los mecanismos y los principales componentes activos.

Palabras clave: Medicina persa; Párkinson; Neurodegeneración; Canela; Ácido cinámico; Cinamaldehído

INTRODUCTION

Parkinson's disease (PD) is ranked as the second common neurodegenerative disorder which is caused due to the dopaminergic neuronal loss in substantia nigra. The mean age of PD onset is 65 years and the prevalence is higher in older people (Gopalakrishna & Alexander, 2015). The main symptoms include motor abnormalities such as bradykinesia, tremor, imbalance, and rigidity (Shahpiri *et al*., 2016), as well as non-motor complications like depression, anxiety, apathy, sleep disorders, gastrointestinal problems, and erectile dysfunction (Chaudhuri & Schapira, 2009). Current pharmacotherapy of PD mainly include dopamine analogue (levodopa), catechol-O-methyl transferase (COMT) inhibitors (entacapone), and monoamine oxidase (MAO)-B inhibitors; however, they show mild to moderate effect on the quality of life of PD patients (Martinez-Martin *et al*., 2015). Thus, investigations to discover new medicines for PD are still necessary.

Dopaminergic neuronal loss in substantia nigra of patients with PD occurs due to different mechanisms. Lewy-bodies, cellular inclusions in neuron mainly consist of α-synuclein, are characteristics of neurons damaged during PD. The physiological role of α-synuclein is to regulate neurotransmitters release in synapses; however, mutations in this protein causes the formation of pathologic forms like fibrils and oligomers which triggers neuroinflammation, dopamine metabolism, oxidative stress in mitochondria and endoplasmic reticulum, and abnormal autophagy (Zeng *et al*., 2018). Another contributor in this process is leucine-rich repeat kinase 2 (LRRK2), a neuronal enzyme, mutation of which results in impaired dopamine signaling and apoptosis (Zeng *et al*., 2018). High kinase activity of mutated LRRK2 stimulates pro-apoptotic mechanisms such as caspase enzymes which causes dopaminergic neuronal loss and manifestations of PD (Ho *et al*., 2009). PTEN-induced putative kinase (PINK) 1, a protein kinase in mitochondria, and its downstream cascade Parkin-ubiquitin, are other participants in degeneration of dopaminergic neurons. Mutations and abnormal activity of these pathways results in mitochondrial dysfunction and is responsible for many of the early-onset PD cases (Zeng *et al*., 2018).

Considering the pathogenesis of PD, several natural therapies are recommended for the management of the condition. The role of oxidative stress in PD pathogenesis is widely accepted and thus, medicinal plants and their active constituents may be promising treatment options due to the high antioxidant power (Wei *et al*., 2018). Polyphenolic compounds, such as flavonoids, are one of the well-known categories of phytochemicals which has demonstrated neuroprotective effects in preclinical models of dopaminergic neuronal loss, mostly due to their antioxidant and anti-inflammatory activities (Shahpiri *et al*., 2016; de Andrade Teles *et al*., 2018). Beneficial effects of other phytochemical categories such as iridoids and alkaloids are also revealed in PD (Shahpiri *et al*., 2016; Dinda *et al*., 2019). Additionally, there is a growing number of clinical and experimental reports on the role of different complementary and alternative medicines such as traditional Chinese medicine (TCM) and Ayurveda in the management of PD complications (Chen *et al*., 2007; Pathak-Gandhi & Vaidya, 2017).

Persian Medicine is one of the most ancient medical doctrines around the world with several pioneers in neurology (Zargaran *et al*., 2012; Zarshenas *et al*., 2012). Cinnamon, with the scientific name of *Cinnamomum verum* J. Presl (synonym: *Cinnamomum zeylanicum* Nees) is one of the important medicinal plants used in Persian medicine for neurological disorders (Jayaprakasha & Rao, 2011). Cinnamon bark is called as "Darchini" and "Darsini" in the text books of Persian medicine and has a hot and dry nature; thus, is widely indicated in the treatment of diseases with cold and wet humors involved in their pathologies. Cinnamon oil, prepared based on the instructions of Persian medicine text books, is useful for neurological problems such as tremor, neuropathies, and headaches (Aghili, 1771). Today's knowledge also supports the beneficial effects of cinnamon in several neurological problems such as Alzheimer's disease (Peterson *et al.*, 2009; Anderson *et al*., 2013) and Parkinson's disease (Shaltiel-Karyo *et al*., 2012; Khasnavis & Pahan, 2014). Aqueous extract of cinnamon bark has shown alleviating effects on the behavioral impairment of experimentally-induced PD in mouse (Mehraein *et al*., 2017). Also, it has demonstrated inhibitory effect on α-synuclein aggregation, a protein involved in the pathogenesis of PD (Shaltiel-Karyo *et al*., 2012). The protective properties of cinnamon purified ingredients against neurodegeneration has been proved in *in vitro* studies (Momtaz *et al*., 2018; Bae *et al*., 2018). The most important phytochemicals identified in cinnamon include cinnamate, cinnamaldehyde, and cinnamic acid (Rao & Gan, 2014). Rat pheochromocytoma (PC-12) cells are a common *in vitro* model for various

neurodegenerative disorders since they represent characteristics of dopaminergic neurons. Induction of cellular damage by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in PC-12 cells is considered as a popular *in vitro* model for PD (Wiatrak *et al*., 2020). Accordingly, the aim of the present study is to evaluate the protective effects of cinnamon oil, prepared according to Persian medicine texts, in MPTP-induced neurodegeneration in PC-12 cells. These treatments were considered regarding their effects on cellular apoptosis, oxidative stress, activity of caspases as pro-apoptotic enzymes, and cell cycle.

MATERIAL AND METHODS

Preparation of cinnamon oil

The dried bark of cinnamon was purchased from a local herbal shop in Isfahan, Iran and was authenticated by botanist as *Cinnamomum verum* J. Presl. A voucher specimen (No. PMP- 908) was deposited in the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Cinnamon oil was prepared according to the instructions in Persian medicine documents (Aghili, 1771). Briefly, 500 g of grinded cinnamon bark was soaked in 3 kg of sesame oil and was exposed to sunlight for 40 days. Then, the oil was filtered and used as traditional cinnamon oil in this study. The final product was subjected to high-performance liquid chromatography (HPLC) for phytochemical analysis.

Quantification of Cinnamaldehyde by HPLC method

Cinnamaldehyde, as the main reported component of the cinnamon bark was chosen to be quantified in the oil. HPLC analysis was performed using the external standard method Shimadzu 10AD high-performance liquid chromatography (Shimadzu, Japan), with a thermostatically-controlled column oven, a binary pump, and a UV/Vis detector. A reversed-phase column Waters ODS C18, 5 µm, 250×4.6 mm (Waters, Ireland), was used in this study. The mobile phase consisted of a gradient of deionized water (solvent A) and methanol (Merck, Darmstadt, Germany) (solvent B) started with 5% B at 0^{th} min, which increased to 18% at 5^{th} min, 35% B at 10^{th} min, 62% B at 15^{th} min, 80% B at 18^{th} min, remained at 80% B to 22^{nd} min, returned to 5% B at 23^{rd} min, and equilibrated at this condition (5%B) for 3 min at 30°C. The flow rate was 1.0 mL/min (injection volume 10 µL) with detection at 280 nm. Prior to injection, cinnamon oil was extracted by addition of methanol and centrifugation at 3500 rpm for 20 min in order to obtain a clear solution for injection. Cinnamaldehyde was identified according to retention time as a comparison with the standard. The concentration of cinnamaldehyde was calculated considering the peak area using calibration curves. According to the guidelines of International Committee of Harmonization (ICH), repeatability and reproducibility of the method was assessed by daily triplicates injection of the samples in three different days.

Cell toxicity assay

Rat pheochromocytoma PC-12 cells (ATCC No. CRL-1721) were purchased from Pasture Institute (Tehran, Iran), and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in 25 cm^2 flasks (SPL Life Sciences Co, Korea). The cells were then treated with 1 mM of MPTP (≥98%, SIGMA, Germany).

Cinnamon oil (M1), cinnamaldehyde (M2, Sigma) and cinnamic acid (M3, Sigma) were dissolved using 1% dimethyl sulfoxide (DMSO) and were diluted into the concentrations of 25, 50, 100, and 200 µg/mL for the primary evaluation of their cytotoxic effects on PC-12 cells. Cells were incubated with M1-M3 for 24 h at 37°C. To determine the toxicity of the compounds, methylthiazol tetrazolium (MTT) assay was carried out 24 h after the incubation period using 100 µL MTT solution for further 2 h at 37°C. The supernatant medium was then removed and formazan grains were washed with 100 µL of DMSO. The absorbance of the wells was measured using an ELISA microplate reader (ELX 800, BioTek) at 490 nm (Akbarzadeh *et al*., 2018).

Since non-toxic concentrations of M2 showed no cytoprotective effect on MPTP-induced neurotoxicity in PC-12 cells (data not shown), this compound was excluded from the rest of the study and the experiments were continued with M1 and M3. Also, the non-toxic concentration of M1 and M3 (200 µg/mL) was chosen as the best concentration which was used for further experiments.

Assessment of ROS formation

Intracellular reactive oxygen species (ROS) formation was assessed using cell permeable 2′,7′-

dichlorofluorescin diacetate (DCFH-DA). In a 12-well plate, cells were cultured (5×10^5 cells in each well) for 24 h. PC12 cells were treated with M1 and M3 in 6-well tissue culture plates for 24 h. After that, the IC_{50} concentration of MPTP was added and the cells were incubated for another 24 h. Intracellular ROS reacts with DCFH-DA and turns it into the oxidized form which has a fluorescent luminescence. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS) and DMEM medium (containing 5% FBS) was added. After another incubation for 24 h, DCFH-DA was added to the cell culture for 45 min at 37°C. The cells were washed with PBS, trypsinized and the supernatant fluid was removed after 10 min centrifugation at 1000 rpm. PBS was again added to the cells and fluorescence intensity was measured with a NovoCyte benchtop Flow Cytometer (San Diego, USA) (Akbarzadeh *et al*., 2018).

Assessment of caspase activity

The assessment of caspase (Casp) activity was conducted on different groups of cells: 1) Control group, 2) Cells treated with MPTP (1 mM) for 2 h, and 3) Cells pretreated with non-toxic concentrations of M1 and M3 (selected after MTT assay) for 24 h and subsequently, treated with MPTP (1 mM) for another 2 h. Then, the cells were washed with PBS, followed by the addition of DMEM-F12 with 5% FBS and incubation for 24 h. The cells were trypsinized, centrifuged at 1400 rpm for 7 min, the supernatant fluid was washed and the cells were subjected to 30 µL lysis buffer. Afterward, the cells were incubated on ice for 30 min and were centrifuged at 14000 rpm for 12 min. The amount of 5 µL of the supernatant liquid was mixed with 30 ml of 1% reaction buffer/dithiothreitol (DTT) and reacted with 5 µL of 1 mM Casp-3 colorimetric substrate. The solution was kept for 2 h at 37°C and the light emission of *p*-nitroaniline was measured by microplate reader at 405 nm wavelength. Bovine serum albumin (BSA) was also used as the standard to measure protein concentration for the equalization of the evaluations according to Bradford method. The same procedures were repeated for the measurement of Casp-8 and Casp-9 activities (Akbarzadeh *et al*., 2018).

Cell cycle analyses

In a 12-well plate, cells were cultured (5×10^5 cells in each well) for 24 h. PC12 cells were treated with M1 and M3 in 6-well tissue culture plates for 24 h. After that, the IC_{50} concentration of MPTP was added to the cells and incubated for another 24 h. Next, the cells were incubated for 2 h at 4°C in dark with 750 µL of a hypotonic buffer (50 mg/mL PI in 0.1% sodium citrate, 100 µg/mL plus 0.1% triton X-100) before flow cytometric analysis using Partec TM cytometry (Germany).

Statistical analysis

All experiments were done in triplicates. Statistical analysis was performed using the Graphpad Prism (version 3.2) and one-way analysis of variances (ANOVA), followed by Tukey's post hoc test. *p* values lower than 0.05 were considered as statistically significant difference.

RESULTS

HPLC quantification of cinnamaldehyde in cinnamon oil

Chromatogram obtained from HPLC measurement of cinnamaldehyde in cinnamon oil is shown in Figure No. 1. The retention time (Rt) of cinnamaldehyde was 12.2 min. Area under the curve (AUC) of the peak was used for calculation. The analysis showed that there was 2 ± 0.09 mg/g cinnamaldehyde in the cinnamon oil.

The calibration curve for the determination of cinnamaldehyde in oil sample is linear over the range of approximately 0.402 to 2.169 µg/mL with a coefficient of determination (r^2) of 0.9999 and equation y = 1E+ 06x. The recovery of cinnamaldehyde was between 99 and 105%.

Protective effects of cinnamon oil and cinnamic acid against MPTP-induced cytotoxicity

PC-12 cells were pre-treated with the toxic concentration (1 mg/mL) of MPTP. Exposure of the cells to MPTP decreased cell viability to 59.46% in comparison to the control cells which treated with normal saline with a *p* value <0.01 (Table No. 1). The highest non-toxic concentration of M2 was 200 µg/mL which showed no significant protective effect on MPTP-induced cytotoxicity. Pre-treatment of PC-12 cells with M1 and M3, followed by the addition of MPTP, could significantly increase cell viability in comparison to MPTP-treated cells ($p<0.05$).

Effect of cinnamon oil and cinnamic acid on ROS formation

Free radicals such as ROS are important contributors of cellular damage. In line with the results obtained from MTT assay, MPTP induced a dramatic rise in

the level of cellular ROS in PC-12 cells (p<0.001). Pretreatment of cells with M1 and M3 could significantly reversed the elevated ROS formation to the normal level with p values lower that 0.05 and 0.01, respectively (Table No. 1).

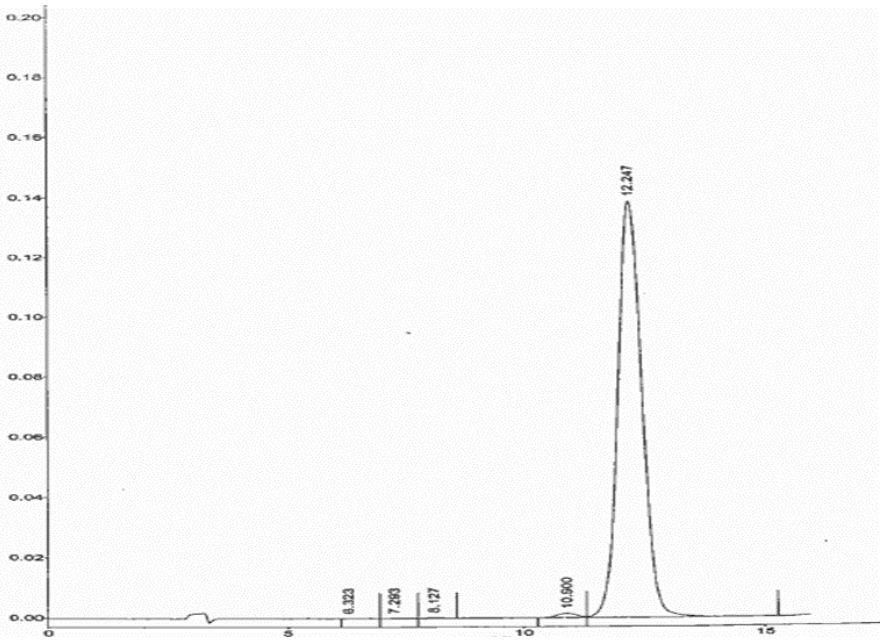

Figure No. 1
Chromatograph of quantification of cinnamaldehyde in cinnamon oil using high-performance liquid chromatography. Measurements showed the presence of 2 ± 0.09 mg/g cinnamaldehyde in the cinnamon oil

Table No. 1
Effect of pretreatment with cinnamon oil and cinnamic acid on cell viability, reactive oxygen species, and caspase enzymes in MPTP-induced cytotoxicity in PC-12 cells

	Viability in MTT assay (%)	ROS (DCF fluorescence)	Casp-3 (%)	Casp-8 (%)	Casp-9 (%)
Control	100±8.59	5553.33±383.65	100.17±1.14	97.26±1.29	100.37±8.03
MPTP	59.46±1.60$^{\#\#}$	8936±373.87$^{\#\#\#}$	149.81±14.77$^{\#\#\#}$	156.41±5.15$^{\#\#\#}$	145.92±6.44$^{\#\#}$
MPTP+M1	105.14±2.68*	5811.67±760.15*	132.95±5.86*	165.07±36.14	71.81±3.50**
MPTP+M3	87.5±4.49*	4873.33±307.59**	90.67±7.68*	85.78±5.65***	79.67±7.48**

Data are expressed as mean ± SEM. Abbreviations: MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, M1: cinnamon oil, M3: cinnamic acid, MTT: methylthiazol tetrazolium, Casp: caspase, ROS: reactive oxygen species, DCF: dichlorofluorescein, $^{*}p$<0.05 vs MPTP, **=p<0.01 vs MPTP, ***=p<0.001 vs MPTP, $^{\#\#}$=p<0.01 vs control, $^{\#\#\#}$=p<0.001 vs control.
Treatment of PC-12 cells with MPTP caused a remarkable increase in apoptosis, ROS formation, and caspase activity in comparison to control; while pretreatment with M1 and M3 could significantly restore these abnormalities.

Effect of cinnamon oil and cinnamic acid on caspase activity

Caspases are a group of enzymes with a critical role in apoptosis. It is demonstrated that these enzymes have a critical role in the development of neurodegeneration during PD (Zeng *et al*., 2018). Addition of MPTP to the media of PC-12 cells increased the activity of different types of Casp enzymes. Regarding Casp-3, MPTP caused a significant rise in the activity of this enzyme ($p< 0.001$) in comparison to control cells. Treatment with M1 and M3 could significantly decrease this abnormal rise ($p<0.05$) and M3 was numerically (but not statistically) more effective than M1. MPTP also caused a dramatic increase in Casp-8 activity ($p<0.001$ vs. control). Although pretreatment with M1 was not effective on this rise, M3 could remarkably ($p<0.001$) normalize Casp-8 activity (Table No. 1). In case of Casp-9, MPTP induced a significant elevation ($p<0.01$) in the enzyme activity. Pretreatment with both M1 and M3 could significantly suppress this abnormal increase with nearly the same potency ($p<0.01$ for both agents).

Effect of cinnamon oil and cinnamic acid on apoptosis

Flow cytometry results showed that the fraction of cells in sub-G1 phase were significantly increased from around 12% in the control group to over 60% in MPTP-treated cells (Figure No. 2). M1 and M3 could successfully reduce sub-G1 cells to lower than 50% which demonstrates the effect of these compounds on the reduction of apoptosis (Figure No. 2).

DISCUSSION

In this study, for the first time, the protective effect of cinnamon oil- prepared according to the instructions of Persian medicine - was evaluated in MPTP-induced neurodegeneration. In our study, cinnamaldehyde and cinnamic acid were primarily considered as main components of cinnamon oil; however, phytochemical analysis using HPLC method showed a low level of cinnamic acid which was not measurable in the cinnamon oil.

In the *in vitro* assessments, non-toxic concentrations of cinnamaldehyde failed to show protective effects against MPTP-induced neurotoxicity. On the other hand, cinnamon oil and cinnamic acid were both effective to reduce the neurotoxic effects of MPTP on PC-12 cells. Previous studies have also assessed the protective effect of cinnamon and its major components in animal and cellular models of PD. Intraperitoneal administration of cinnamon aqueous extract to the mouse model of MPTP-induced PD with a dose of 40 mg/kg could significantly improve balance and motion, evident form the results of the rotarod test (Mehraein *et al*., 2017). Cinnamaldehyde has demonstrated protective effects against oxygen and glucose deprivation/reperfusion (OGD/R)-induced damage in PC-12 cells through reduction of ROS and apoptosis. Nitric oxide (NO) and phosphatidylinositol 3-kinase (PI3K) are two main pathways involved in the protective effect of cinnamaldehyde in PC-12 cells. Another mechanism suggested by Qi *et al*. (2016), is the improvement of mitochondrial membrane potential which is an indicator of its function and membrane integrity (Qi *et al*., 2016). It is also demonstrated that PINK-1-Parkin-ubiquitin signaling in mitochondria has a critical role in the development of neurodegeneration in PD (Zeng *et al*., 2018). Thus, mitochondrial targets can be considered as future therapeutic targets in the management of PD. A recent publication has reported the neuroprotective effect of two *Cinnamomum* species, as well as cinnamaldehyde, on 6-OHDA-induced apoptosis in PC12 cells. A significant decrease in sub-G1 cells was reported for cinnamon extracts and cinnamaldehyde-treated cells in comparison to 6-OHDA damaged cells (Ramazani *et al*., 2020). This is in line with our findings on cinnamon oil and cinnamic acid, showing a reduction of sub-G1 cell population compared with MPTP-intoxicated cells. In addition to a decrease in ROS formation and apoptosis which is in line with our results, the study has also suggested a role for survivin and cytochrome-C as the subcellular pathways involved in the observed protective mechanisms (Ramazani *et al*., 2020).

Oxidative stress-induced neuronal damage is an important contributor in the pathogenesis of PD (Emerit *et al*., 2004). Apoptosis can be triggered by a variety of extrinsic and intrinsic signals. These include different stresses such as ROS, reactive nitrogen species (RNS), DNA-damaging agents (e.g. radiation), heat shock, serum deprivation, viral infection and hypoxia (Zhang *et al*., 2013). ROS is a physiologic by-product of electron transfer chain in mitochondria; however, its overproduction causes a pathologic condition which leads to mitochondrial dysfunction. Malfunction of mitochondrial complex I results in excessive ROS formation and adenosine triphosphate (ATP) shortage which seems to be responsible for the signs and symptoms in about 30%

of patients with PD (Motherwell & Zuo, 2013). Phosphorylation of P44/42, a mitogen-activated protein kinase (MAPK), occurs in response to ROS overproduction. Subsequently, mitochondrial cytochrome-C is released into cytoplasm and forms apoptosomes, followed by activation of Casp-9, as well as other caspases. Thus, overproduction of mitochondrial ROS acts as a trigger of both caspase-dependent and caspase-independent apoptosis (Dias *et al*., 2013; Ramazani *et al*., 2020). In addition to mitochondria, other cellular organelles such as endoplasmic reticulum are also involved in ROS production. Excessive ROS leads to structural defects of lysosomes and abnormal membrane permeability (Motherwell & Zuo, 2013). Our study showed that both cinnamon oil and cinnamic acid can significantly prevent MPTP-induced production of ROS in PC-12 cells. As mitochondria plays an essential role in the pathogenesis of PD due to different stressors, future studies on the effect of cinnamon oil on mitochondrial function are recommended.

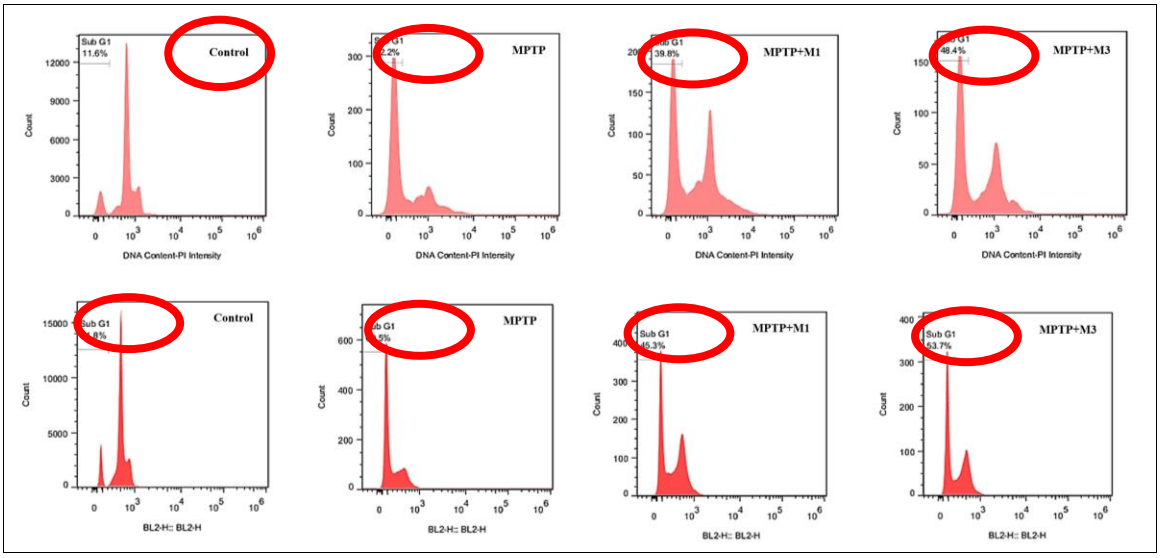

Figure No. 2
Effect of cinnamon oil (M1) and cinnamic acid (M3) on MPTP-induced apoptosis in PC12 cells by flow cytometric DNA analysis. MPTP caused a significant rise in sub-G1 cells in comparison to control; while pretreatment with M1 and M3 could significantly prevent this increase

Our data showed that cinnamon oil and cinnamic acid were able to regulate the activity of different types of Casp enzymes. Caspases are a group of cysteine protease enzymes involved in the programmed cell death. They orchestrate both extrinsic and intrinsic pathways of apoptosis by activating target protein cleavage (Pulikkan *et al*., 2018). It is demonstrated that abnormally activated Casp enzymes participate in the pathogenesis of several neurodegenerative disorders, including PD (Friedlander, 2003). Evaluation of ubiquitin-proteasome system in peripheral blood lymphocytes of patients with neurodegenerative disorders, including PD, showed a lower proteasomal activity in comparison to healthy subjects. This lower activity leads to formation of protein aggregates such as Lewy bodies (Blandini *et al*., 2006). These protein inclusions formed from α-synuclein are specific features of neurodegeneration in substantia nigra of PD patients (Zeng *et al*., 2018). The observed lower proteasomal activity seems to be due to overactivation of Casp-3 and Casp-9, both in brain and non-neuronal cells, which triggers the process of neurodegeneration, resulting in PD (Blandini *et al*., 2006). Increased Casp-3 and Casp-9 activity, along with higher expression of Bax, a proapoptotic factor,

in neurons of PD patients. Also, preclinical studies have demonstrated that reduced proteasomal activity facilitates the activation of proapoptotic cascades such as Caspase enzymes. These data, together, shows the importance of caspase activity in the development of neurodegeneration in PD; thus, their regulation by cinnamon is one of the main neuroprotective mechanisms of this plant in neurodegenerative disorders.

In an *in vivo* study by Bae *et al*. (2018), i.p. administration of cinnamaldehyde was significantly effective on the rat model of MPTP-induced PD (Bae *et al*., 2018). Also, Mehraein *et al.* (2017) reported significant improvement in rotarod performance in MPTP-induced mice pretreated with 30 mg/kg of cinnamaldehyde. The same dose of cinnamaldehyde was effective in the mouse model of 6-hydroxydopamine- induced PD, as well (Pyo *et al*., 2013). These results may seem controversial to our data in first look; however, the key to clarify these data is the pharmacokinetics of cinnamaldehyde in the *in vivo* models. Comparison the molecular structure of cinnamaldehyde and cinnamic acid shows that these molecules are only different in one oxygen atom. Thus, cinnamaldehyde turns to cinnamic acid in an oxidation reaction (Yuan *et al*., 1992). As widely discussed in previous literatures, several enzymes in different parts of body such as liver and blood stream are able to catalyze the oxidation process (Guengerich & Shimada, 1991); thus, a possible explanation for the positive effect of cinnamaldehyde in animal models is that the molecule is oxidized to cinnamic acid which consequently exhibits neuroprotective effects. In other words, cinnamaldehyde acts as a pro-drug which turns to the active form, cinnamic acid, in biological system. The protective effects of cinnamon oil in PC-12 cells may be due to the presence of phytochemicals other than cinnamaldehyde. There are also reports on the protective effect of cinnamaldehyde administration *in vitro* such as in lipopolysaccharide (LPS)-activated BV2 microglia cells (Ho *et al*., 2013), as well as in oxidative damage and apoptosis in high glucose-induced dorsal root ganglial cells of rat (Yang *et al*., 2016), with concentrations of 100 μM and 100 nM of cinnamaldehyde; however, the type of cell lines was different compared with that of our study which can be the reason for different results obtained in those studies.

In cinnamon oil- prepared based on the instructions of Persian medicine- cinnamon bark is extracted in sesame oil as vehicle. Thus, it should be considered that this type of cinnamon oil is a mixture of cinnamon secondary metabolites and sesame oil components such as different unsaturated fatty acids, lignans like sesamin and sesamolin, and tocopherols (Rangkadilok *et al*., 2010) that may affect the penetration of cinnamon compounds and consequently, its pharmacological activity. This may also provide another explanation for our results regarding the ineffectiveness of pure cinnamaldehyde; while cinnamon oil containing cinnamaldehyde was significantly effective. Also, effectiveness of cinnamaldehyde in other *in vitro* models of PD may be due to the different method of disease induction and subsequently, different affected pathways. However, all these hypotheses need to be assessed in future investigations.

There are also some studies which suggest other mechanisms to be involved in the neuroprotective effects of cinnamon. Glial cells are important participants of chronic neuroinflammation (Blesa *et al*., 2015). The role of inflammation in pathogenesis and progression of PD has been demonstrated in previous investigations (Bassani *et al*., 2015). In LPS-activated BV2 microglia cells, cinnamon extract and eight of its major components showed protective effects against neuroinflammation amongst which cinnamaldehyde was the most potent one. Reduction of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, as well as other markers of inflammation such as NO and nuclear factor-κB (NF-κB) were the mechanisms involved in the anti-inflammatory effect of cinnamon (Ho *et al*., 2013). It has long been demonstrated that PD is associated with higher levels of IL-1β and IL-6 in both serum and cerebrospinal fluid of patients and the severity of the patient symptoms correlates with the levels of these cytokines (Blum-Degena *et al*., 1995; Hofmann *et al*., 2009). Suppressing these cytokines upon treatment with cinnamon may result in reduction of neuronal damage and subsequently, lower clinical symptoms in PD patients. Although most studies have focused on cinnamaldehyde as the active ingredient of cinnamon, some studies also suggest sodium benzoate (Khasnavi & Pahan, 2012) and procyanidins (Panickar *et al*., 2012) to be effective in the prevention of neuronal damage and inflammation which may be interesting to be further investigated in future studies. Further mechanistic studies on the neuroprotective effects of cinnamon, as well as pharmacokinetic evaluations are recommended to

provide more details regarding the pharmacology and active ingredients of this plant in PD.

CONCLUSIONS

Our study demonstrated the protective effect of cinnamon oil and cinnamic acid in an *in vitro* model of neurodegeneration. This effect is, at least in part, mediated via the regulation of different Casp enzymes and prevention of oxidative neuronal damage; however, other mechanisms such as anti-inflammatory properties may also be involved which can be the subject of future studies.

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