Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Bioassay-guided Isolation of Neuroprotective Fatty Acids from *Nigella sativa* against 1-methyl-4-phenylpyridinium-induced Neurotoxicity

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Submitted: 21-10-2016 Revised: 02-12-2016 Published: 13-11-2017

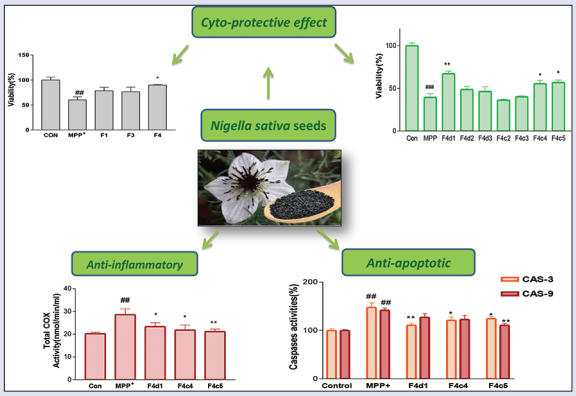
ABSTRACT

Objective: Parkinson's disease, a slowly progressive neurological disease, is associated with degeneration of the basal ganglia of the brain and a deficiency of the neurotransmitter dopamine. The main aspects of researches are the protection of normal neurons against degeneration. Fatty acids (FAs), the key structural elements of dietary lipids, are carboxylic straight chains and notable parameters in nutritional and industrial usefulness of a plant. **Materials and Methods:** Black cumin, a popular anti-inflammatory and antioxidant food seasoning, contains nonpolar constituents such as FAs which were extracted using hexane. Different fractions and subfractions were apt to cytoprotection against apoptosis and inflammation induced by 1-methyl-4-phenylpyridinium (MPP+) in rat pheochromocytoma cell line (PC12) as a neural cell death model. The experiment consisted of examination of cell viability assessment, mitochondrial membrane potential (MMP), caspase-3 and -9 activity, and measurement of cyclooxygenase (COX) activity. **Results:** MPP+ induced neurotoxicity in PC12 cells. Pretreatment with subfractions containing FA mixtures attenuated MPP+-mediated apoptosis partially dependent on the inhibition of caspase-3 and -9 activity and increasing the MMP. A mixture of linoleic acid, oleic acid, and palmitic acid also decreased the COX activity induced by MPP+ in PC12 cells. **Conclusion:** Our observation indicated that subtoxic concentration of FA from *Nigella sativa* may exert cytoprotective effects through their anti-apoptotic and anti-inflammation actions and could be regarded as a dietary supplement.

Key words: Linoleic acid, neuroprotective effect, oleic acid, Parkinson's disease, pheochromocytoma 12 cells

SUMMARY

- MPP+ induced neurotoxicity in PC12 cells
- *Nigella sativa* contains bioactive fatty acids
- Pretreatment with fatty acids attenuated MPP+ mediated apoptosis through inhibition of caspase 3 and 9 activity
- A mixture of linoleic acid, oleic acid, and palmitic acid decreased the COX activity induced by MPP+ in PC12 cells
- Due to cytoprotective, anti apoptotic and anti inflammation actions of *N. sativa*, it could be regarded as a dietary supplement.



Abbreviations used: ANOVA: Analysis of variance; Ca: Calcium; CDCl3: Chloroform; COX: Cyclooxygenase; DMSO: Dimethyl sulfoxide; EA: Elidic acid; EDTA: Ethylene diamine tetraacetic acid; ELISA: Enzyme Linked Immunosorbent Assay; ESI-MS: Electron spray mass spectroscopy; FAs: Fatty acids; FBS: Fetal bovine serum; GC: Gas chromatography; 1HNMR: Hydrogen nuclear magnetic resonance; LA: Linoleic acid; MPP+: 1-Methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; *N. sativa*: *Nigella sativa*; OA: Oleic acid; PA: Palmitic acid; PBS: Phosphate buffer saline; PC12: Rat pheochromocytoma cell line; PD: Parkinson's disease; PDA: Photo diode array detector; PGE2: Prostaglandin E2; TLC: Thin layer chromatography; TMPD: N,N,N′,N′-tetramethyl-p-phenylenediamine; USA: United states of America.

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DOI: 10.4103/pm.pm_470_16

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INTRODUCTION

The progressive loss of structure or function of neurons leads to neurodegenerative diseases such as Parkinson's disease (PD).[1] It is the second most common neurodegenerative disorder and the most common motility disorder.[2] Although it is accepted that lack of dopamine causes the motor symptoms of PD, it is not clear why the dopamine-producing brain cells degenerate. Many biological

Cite this article as: Hosseinzadeh L, Monaghash H, Ahmadi F, Ghiasvand N, Shokoohinia Y. Bioassay-guided isolation of neuroprotective fatty acids from *Nigella sativa* against 1-methyl-4-phenylpyridinium-induced neurotoxicity. Phcog Mag 2017;13:627-33.

studies have revealed that aberrant $Ca2^+$ homeostasis, oxidative stress, mitochondrial dysfunction, apoptosis, and upregulation and activation of $Ca2^+$-dependent cysteine proteases, calpain and caspase-3, can all contribute to neuronal death in PD.[3-5] Therefore, the regulation of the apoptotic cascade may control apoptotic events lead to dopaminergic neuronal cell death. Moreover, neuroinflammation is also a significant aspect of brain injuries and nervous system disorders such as PD, multiple sclerosis, and Alzheimer's disease.[6] Therefore, regulating the inflammatory response could be an important part of the treatment of PD.

1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, is a commonly used neurotoxin to produce neuronal cell apoptosis and neurodegenerative models.[5] It has been used extensively in various studies to produce an experimental model of PD.[3,7]

Herbal medicines, with their wide variety of phytochemical molecules, have revealed their protective and therapeutic effects of neuropsychological diseases. A widespread research demonstrates the role of plant-derived natural molecules in protection and treatment of PD as single or combined with the current pharmaceutical drugs.[8]

Nigella sativa (black cumin) is a traditional medicinal plant endemic to Western Asia which is considered blessing and miracle.[9] Black cumin showed anti-inflammatory effects, and its antioxidant potency has been reported in several experiments,[10,11] especially regarding its neuroprotective activity.[12-15] Thymoquinone, a bioactive phytochemical constituent of the oil of the seeds of *N. sativa*, could afford neuroprotection against 6-hydroxydopamine-induced neurotoxicity.[16] However, to the best of our knowledge, no data have been reported on the neuroprotective effect of *N. sativa* constituents. The present *in vitro* study is designed to bioassay-guided isolation of neuroprotective constituents of hexane extract of the seeds of *N. sativa* on MPP^+-induced cell injury in PC12 cells. PC12 cells were chosen as they can be secreted dopamine neurotransmitters and contain high amounts of dopamine transporters.[17] This cell line is a widely accepted model of neuronal cells,[18] therefore, it is commonly used in the investigation of neurotherapeutics study for PD.[7,19]

MATERIALS AND METHODS

General instruments and chemicals

NMR spectra were measured on a Bruker® (400 MHz) spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl3: δH 7.30). ESI-MS spectra were performed on an Agilent 5973 Network Mass Selective Spectrometer. Open column chromatographies were performed using silica gel (70–230 mesh); separations were monitored by thin-layer chromatography (TLC) on Merck 60 F254 (0.25 mm) plates and were visualized by ultraviolet inspection and/or staining with 0.2% cerium sulfate/4.2% sodium molybdate and heating; high-performance liquid chromatography (HPLC) were performed on a Young Lin apparatus equipped with a pump (YL 9111S) and PDA detector (YL 9160) using Eurospher II (normal phase, Si 250 mm × 20 mm) columns with flow rate as 10 mL/min. Varian CP-3800 GC system equipped with flame ion detector and CP-Sil 88 capillary column (length: 100 m; inner diameter: 0.25 mm). MPP^+, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamin 123, caspase-3 activity assay kit, and caspase-9 substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclooxygenase (COX) activity assay kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Cell culture medium, penicillin-streptomycin, and fetal bovine serum (FBS) were bought from Gibco (Grand Island, NY, USA). All the solvents used for extraction and purification were purchased from Merck (Germany) and Dr. Mojallali (Iran).

Plant material

Seeds of *N. sativa* L. were bought from Faculty of Agriculture, Razi University, where the herbarium specimen was identified by Dr. Nastaran Jalilian and compared to the specimen No. 248008007. For herbarium sample preparation, seeds were grown in proper condition and plant material was dried and pressed.

Bioguided fractionation and purification of active constituents

N. sativa seeds (500 g) were extracted with hexane (2000 ml) using a Soxhlet apparatus for 4 h. The extract (25 g) was soaked in 250 ml MeOH and kept for 48 h at − 20°C and chilled filtered. The filtrate was concentrated *in vacuo* (12 g) and was fractionated by vacuum liquid chromatography (sorbent, silica gel) using a gradient of hexane and EtOAc from 100% of the premier to 0%, to afford five fractions (F1–F5) based on TLC profile.

Fraction F4, which was the most active fraction in biological tests, as will be explained in the results section, was further purified using another normal phase column with hexane in EtOAc (80%–20%) to get four subfractions as F4a–F4d. Subfractions were purified using normal phase HPLC with hexane/EtOAc solvent system to get two series of compounds, one F4d1–F4d3 and the other F4c1–F4c5 [Figure 1]. To identify structures, samples were sent for spectroscopic analyses. Since the active constituents determined to be fatty acids (FAs), the profile of FAs in total extract was determined through GC/FID system as well.

Cell culture

PC12 cells, a clonal cell line derived from a pheochromocytoma of the rat adrenal medulla cancer cells, were cultured in a medium containing 10% FBS which includes 100 U/ml penicillin and 100 U/ml streptomycin and L-glutamine and were kept in an incubator with 37°C and 5% CO_2.

Viability assay

Four sets of experiments were performed at standard culture conditions: (1) untreated control cells, (2) cells were treated with different concentrations of extract, fractions, and subfractions, (3) cells were treated with MPP^+ (1.5 mM), and (4) cells were pretreated with different concentrations of fractions and subfractions for 24 h, and then medium was changed and cells were treated with 1.5 mM concentration of MPP^+ for another 24 h. Briefly, after treatment, the medium was removed and replaced with 200 μl of 0.5 mg/ml of MTT in growth medium and then the plates were transferred to a 37°C incubator for 3 h. Later, the medium was removed, and the purple formazan crystals were dissolved in DMSO (200 μl/well). Absorbance was determined on an ELISA plate reader (Biotek, H1M), with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630).

Caspase-3 and -9 activation assay

To investigate the activity of caspase-3 and -9 enzymes, around 1×10^6 cells are collected and lysed using cold lysis buffer and are kept on ice for 10 min. Afterward, lysed cells are centrifuged for 5 min at a temperature of 4°C and before adding the substrate of caspase-3 (Ac-DEVD-pNA) and caspase-9 (Ac-LEHD-pNA). To measure the caspases activity, the amount of protein per sample is assessed through Bradford method and using Coomassie Brilliant Blue which is bonded to protein in the acidic environment and produces a red color. After adding the substrate of caspase-3 and -9, it was incubated for 1 h at 37°C and then light absorption of pNA was measured at a wavelength of 400–405 nm using the spectrophotometer. Comparing pNA absorbance of the apoptotic sample with control sample indicates the rate of caspase activity.

Mitochondrial membrane potential assay

The fluorescence color of rhodamine 123 (Sigma-Aldrich, Germany) was used for the measurement of mitochondrial membrane potential (MMP). First, the fluorescence color of rhodamine 123 with a concentration of 20 mM was added to the cells, and the cells are incubated for ½ hour at 37°C. Then, the cells are washed with phosphate-buffered saline (PBS), and the amount of their fluorescence is measured at a wavelength of 488–510 nm using fluorescence microplate reader.[20]

Cyclooxygenase activity assay

PC12 cells were harvested and the proteins were extracted from 1×10^6 cells using the lysis cold buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM EDTA). Then, the protein content was determined by the Bradford method using the bovine serum albumin as a standard and the protein concentrations were equalized for each condition. Next, the COX activity assay was measured in the proteins extracted from PC12 cells by Cayman COX Activity Assay Kit (Cayman Chemical Company) according to the

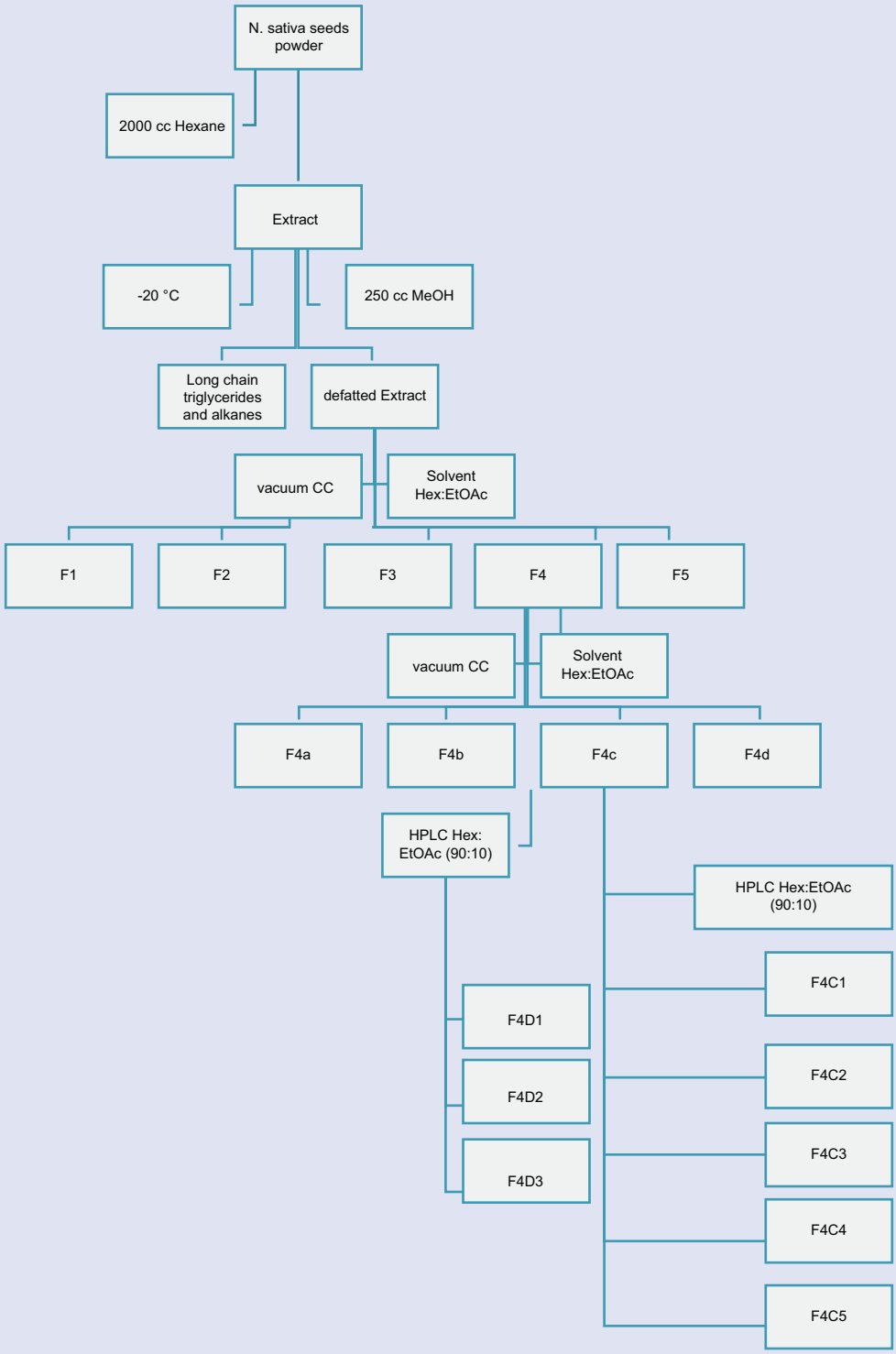

Figure 1: The schematic flowchart of purification of *Nigella sativa* seed extract

manufacturer's procedure. Cayman's COX Activity Assay Kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N, N, N', N'-tetramethyl-p-phenylenediamine at 590 nm.

Statistical analysis

Prism software is used for analyzing the data related to protective effects and IC50 calculation. Mean and standard deviation are calculated for all the parameters. To examine the mean difference of groups, INSTAT statistical program and one-way ANOVA test and the relevant posttest are used.

RESULTS AND DISCUSSION

Effect of fractions and subfractions pretreatment on 1-methyl-4-phenylpyridinium-induced cell death

To exclude the possibility that fractions their self, affected the survival of PC12 cells, different concentrations of subfractions (0–50 μg/cc) were added to the cells for 24 h. Our MTT results showed that the F1, F3, and F4 fractions are not able to induce cytotoxicity in PC12 cells at the concentration up to 50 μg/ml [Figure 2]. Therefore, these fractions were selected for the next study. To further determine the neuroprotective effects of F1, F3, and F4 fractions on MPP^+-induced cell deaths, PC12 cells were treated for 24 h with 50 μg/ml of F1, F3, and F4 fractions before exposure to 1.5 mM of MPP^+. Figure 3 shows a significant decrease in the cell viability in MPP^+-induced group as compared to control group ($P < 0.01$) and significant increase of the cell viability ($P < 0.05$) in MPP^+-induced groups treated with F4 fraction as compared to MPP^+ group. Based on this information, fraction F4 was further purified in three steps to get eight subfractions. As shown in Figure 4a, except for F4c2, all subfractions had no significant effect on the PC12 cell viability up to 200 μg/ml. When the effect of nontoxic concentrations of subfractions was evaluated on cell injury induced by MPP^+, our results showed that cytotoxic effects of MPP^+ on PC12 cells were significantly blocked by pretreatment with F4d1, F4c4, and F4c5 [Figure 4b]. Therefore, in subsequent experiments, these subfractions were selected for the identification of mechanisms of cytoprotective action in the PC12 cell line.

Effects of fatty acid mixtures on mitochondrial membrane depolarization (mitochondrial membrane potential) induced by 1-methyl-4-phenylpyridinium

To characterize the changes in mitochondrial events induced by MPP^+ and/or selected subfractions treatments, the collapse of MMP in PC12 cells was monitored with a cell permeable cationic fluorescent dye, rhodamine 123. Fluorescence intensity was dramatically reduced after incubating cells with 1.5 mM of MPP^+ for 24 h ($P < 0.001$). F4c5 subfraction attenuated the MMP changes in MPP^+-treated PC12 cells. Compared with the group treated with MPP^+ alone, fluorescent intensities increased significantly after the use of F4c5 pretreatments ($P < 0.01$) [Figure 5].

Effect of fatty acid mixture pretreatment on caspase-3 and -9 activity

After 24 h treatment of PC12 cells with 1.5 μM MPP^+, a 43% increase in caspase-3 and 39% increase in caspase-9 activity were detected in comparison to control. In contrast, PC12 cells that were treated with F4d1, F4c4, and F4c5 exhibited a significant decrease in caspase-3 activity compared with the MPP^+-treated cells at the same time point [Figure 6]. Our results also showed a significant decrease in caspase-9 activity after pretreatment with F4c5 ($P < 0.05$) [Figure 6].

Effect of fatty acid mixture pretreatment on cyclooxygenase activity

To gain further insight into the action of subfractions, which supported the cell survival under the MPP^+-induced neuropathological stress, the activity of COX was assessed. Treatment of PC12 cells with MPP^+ significantly increased COX activity after 24 h of exposure in comparison to control. As it can be observed in Figure 7, this increase was attenuated by pretreatments of F4d1, F4c4, and F4c5.

Structure elucidation of bioactive constituents

The structures of the phytochemicals which were the most effective in biological tests were elucidated based on NMR, mass, and GC data.[21-23] F4d1 to F4d3 and F4c1 to F4c5 were determined to be FA derivatives in different ratios. Active fractions are as follows: F4c4 consisted of linoleic acid (LA) 40%, oleic acid (OA) 22%, palmitic acid (PA) 14%, and elaidic acid (EA) 11%; F4c5 consisted of LA 43%, OA 21%, PA 13%, and EA 7%; and F4d1 consisted of LA 52%, OA 25%, and PA 16%.

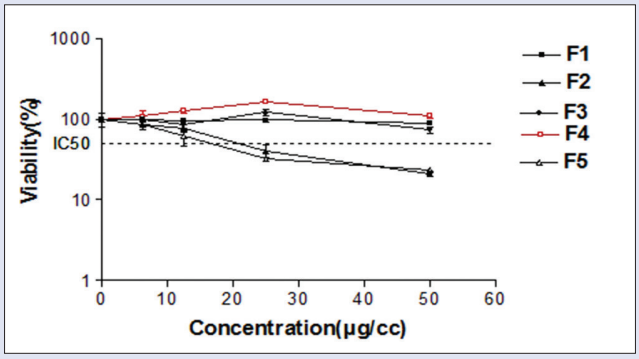

Figure 2: Cell viability of PC12 cells after exposure to different concentrations of fractions (0–50 μg/ml) from *N. sativa* hexane extract. Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as the mean ± standard error of the mean of three separate experiments ($n = 3$)

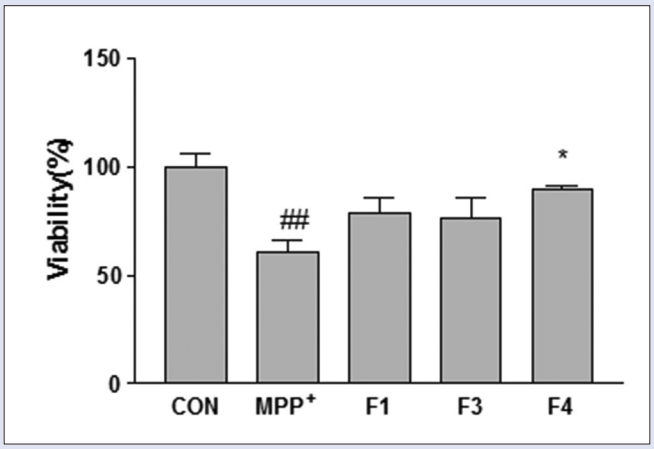

Figure 3: F4 prevented the cell viability decrease induced by MPP^+. PC12 cells were pretreated with F1, F3, and F4 for 24 h followed by incubation with MPP^+ (1.5 mm) for 24 h. The cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay as described in materials and methods. Data are expressed as the mean ± standard error of the mean of three separate experiments. MPP^+: 1-methyl-4-phenylpyridinium. $^{\#\#}P<0.01$ versus control, $^{*}P<0.05$ versus MPP+ treated cells

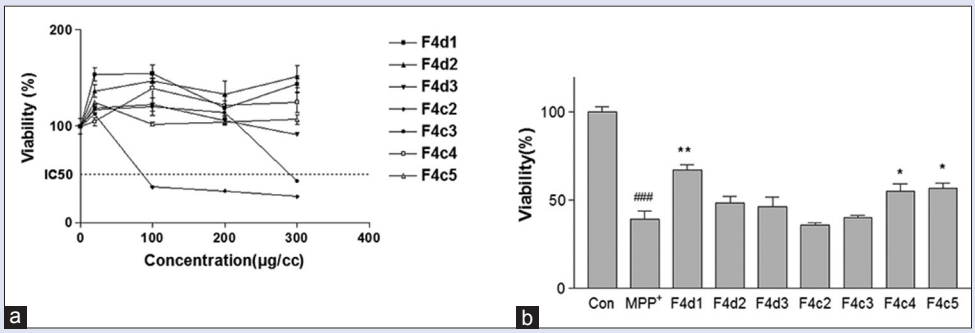

Figure 4: (a) The effects of subfractions (0–200 µg/ml) purified from F4 of *Nigella sativa* hexane extract on the viability of PC12 cells. (b) The effects of nontoxic concentration of subfractions against cytotoxicity induced by MPP+. Incubation of PC12 cells with MPP+ (1.5 mM) induced a decrease in cell viability, while F4d1, F4c4, and F4c5 subfractions had protective effect. The cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay as described in materials and methods. Data are expressed as the mean ± standard error of the mean of three separate experiments. ###$P < 0.001$ versus control, **$P < 0.01$, *$P < 0.05$ MPP+ treated cells. MPP+: 1-methyl-4-phenylpyridinium

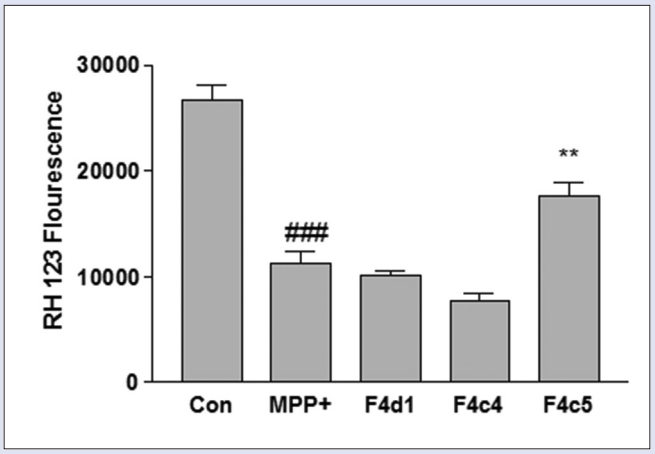

Figure 5: Effect of F4d1, F4c4, and F4c5 subfractions purified from F4 fraction of *Nigella sativa* on MPP+ (1.5 mM)-induced mitochondrial membrane potential collapse as detected by rhodamine 123. Data are expressed as the mean ± standard error of the mean of three separate experiments. ###$P < 0.001$ versus control, **$P < 0.01$ versus MPP+ treated cells. MPP+: 1-methyl-4-phenylpyridinium

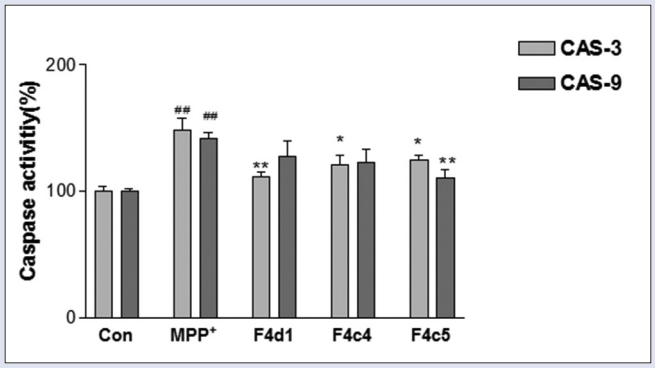

Figure 6: Effect of F4d1, F4c4, and F4c5 subfractions purified from F4 fraction of *Nigella sativa* hexane extract on caspase-3 and -9 activity. Cell pretreated with subfractions 24 h before exposure to MPP+. Caspase-3 and -9 activity was measured by colorimetric detection of p-nitroaniline and expressed as percent of control. Data are expressed as the mean ± standard error of the mean of three separate experiments. ##$P < 0.01$ versus control *$P < 0.01$ versus MPP+ treated cells. MPP+: 1-methyl-4-phenylpyridinium. *$P<0.05$, **$P<0.01$ versus MPP+ treated cells

Total FA profile of the hexane extract is as follows: LA (37.35%), OA (30.68%), PA (26.22%), myristic acid (3.4%), and EA, C17, stearic acid, eicosadienoic acid, caproic acid, etc., (2.35%).

Linoleic acid

^{1}HNMR ($CDCl_3$, 400 MHz) δ_H 0.92 (3H, H_{18}, t, J = 1.2 Hz), 1.22 (8H, H_4, H_5, H_6, H_{16}, d), 1.35 (4H, H_7, H_{15}, d), 1.67 (4H, H_3, s), 2.08 (4H, H_8, H_{14}, m), 2.38 (2H, H_2, m), 2.81 (2H, H_{11}, t), 5.40 (4H, H_9, H_{10}, H_{12}, H_{13}, m). EI MS: m/z 280 $[M]^+$, 206 $[M-74]^+$, 164 $[M-116]^+$, 249 $[M-31]^+$, m/z = 74.

Oleic acid

^{1}HNMR ($CDCl_3$, 400 MHz) δ_H 0.92 (3H, H_{18}, t), 1.34 (20H, H_4, H_5, H_6, H_7, H_{12}, H_{13}, H_{14}, H_{15}, H_{16}, H_{17}, m), 1.64 (2H, H_3, m), 2.02 (2H, H_8, H_{11}, m,), 2.38 (2H, H_2, t), 5.39 (4H, H_9, H_{10}, t). EI MS: m/z 282 $[M]^+$, 208 $[M-74]^+$, 166 $[M-116]^+$, 250 $[M-32]^+$, m/z = 74.

Palmitic acid

^{1}HNMR ($CDCl_3$, 400 MHz) δ_H 0.92 (3H, H_{16}, t), 1.34 (22H, H_4, H_5, H_6, H_7, H_8, H_9, H_{10}, H_{11}, H_{12}, H_{13}, H_{14}, m), 1.41 (2H, H_{15}, t), 2.38 (2H, H_2, t). EI MS: m/z 256 $[M]^+$, 182 $[M-74]^+$, 213 $[M-43]^+$, 225 $[M-31]^+$, m/z = 87, 101, 115, 129, 143, 157, 199, etc., of general formula $[CH_3OCO(CH_2)_n]^+$, m/z = 74.

Elaidic acid

^{1}HNMR (CDCl3, 400 MHz) δH 0.92 (3H, H18, t), 1.34 (20H, H4, H5, H6, H7, H12, H13, H14, H15, H16, H17, m), 1.64 (2H, H3, m), 2.02 (2H, H8, H11, m), 2.38 (2H, H2, t), 5.39 (4H, H9, H10, t). EI MS: m/z 282 $[M]^+$, $[C_nH_{2n-1}]^+$, 264 $[M-18]^+$, 60 (McLafferty rearrangement ion) $[M-222]^+$.

Based on HNMR data, the active fractions contain FA derivatives. A peak at δ 5 to 6, representing unsaturated FA, along with other common features such as triplet at *ca*. δ 0.9 (terminal CH3), hump of hydrogen resonance at δ 1.5-2 (homolog CH_2 series (CH_2) n), and several peaks at δ 1.7–2.8 related to more deshielded methylenes. According to the retention time of compounds in GC column compared to those of FA standards, the constituents were identified and further authenticated based on EI-mass spectra.

In mass spectra of unsaturated FA, molecular ions $[C_nH_{2n-1}]^+$, an ion representing the loss of water from the carboxyl group ($[M-18]^+$), i.e., 264

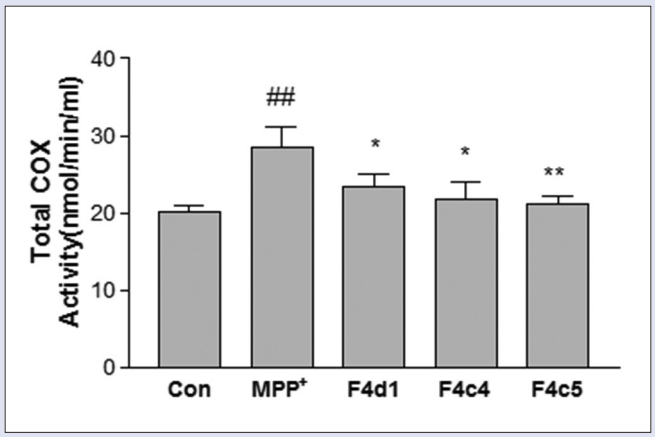

Figure 7: Effect of F4d1, F4c4, and F4c5 subfractions purified from F4 fraction of *Nigella sativa* hexane extract on cyclooxygenase activity. Cell pretreated with subfractions 24 h before exposure to MPP^+. Data are expressed as the mean ± standard error of the mean of three separate experiments. $^{\#\#}P < 0.01$ versus control, $^{*}P < 0.05$, $^{**}P < 0.01$ versus MPP^+ treated cells. MPP^+: 1-methyl-4-phenylpyridinium

for OA, and homolog ions $[55 + CH_2)\ n]^+$, are abundant. The McLafferty ions are small peaks instead. On the other hand, in mass spectra of saturated ones, the molecular ion is clearly abundant with other peaks such as $[M-17]^+$, McLafferty rearrangement ion at m/z 60 and 73, and homolog fragments obtained from methylene lose from $[HOOC (CH_2)\ n]^+$ ranged m/z 115–255.

DISCUSSION

PD is a chronic, neurodegenerative disorder that results from the gradual and progressive loss of dopaminergic neurons in the *substantia nigra*. Many before studies demonstrated that MPP^+-induced cell injury is a useful representation for studying dopaminergic degeneration modeling PD both *in vitro* and *in vivo*.[7,24] PC12 cells are suitable *in vitro* model for Parkinson and DA neurons studies. PC12 cells possess many of the characteristics of human neurons and therefore are suitable *in vitro* model for Parkinson and DA neurons studies.[7,24] This study was drawn to test a series of FAs such as LA and OA isolated from hexane extract of the seeds of *N. sativa*, as protective agents against neuronal damage induced by MPP^+ in PC12 cells. Here, we confirmed that 1.5 mM of MPP^+ caused a significant reduction in cell viability. Our results also showed that subfractions F4d1, F4c4, and F4c5 proved to have a potent protective effect on cell injury induced by MPP^+. These fractions contain LA, OA, and PA in different ratios in which the premier is in a higher amount. LA has two double bonds apt to be oxidized and be an antioxidant. Mousavi *et al*. have previously shown that ethanolic extract of *N. sativa* is able to protect PC12 cells from death induced by serum/glucose deprivation.[25]

In the next set of our experiment, therefore, to gain an understanding of the mechanisms by which these compounds can protect PC12 cells from death induced by MPP^+, we examined some parameters involved in apoptosis. Mitochondria play key roles in activating apoptosis in mammalian cells. A key feature of mitochondrial apoptosis is disruption of the membrane potential mainly caused by increased membrane permeability.[26] Permeabilization of the mitochondrial membrane causes bioenergetics failure and permits the release of soluble molecules from the outer space of the mitochondria to the cytosol, ultimately leading to cytochrome C release and caspase-9 activation.[27] Mature caspase-9 activates additional caspase-9 molecules as well as caspase-3, in turn, the execution of cell apoptosis. In turn, caspase-3 activates downstream caspases in a proteolytic cascade.[28] In agreement with previous studies, MPP^+ treatment caused a significant decrease in MMP levels and also increase in caspase-9 and -3 activation;[29,30] our results revealed that pretreatment with F4d1, F4c4, and F4c5 attenuated caspase-3 activity. Moreover, we showed that F4c5 is able to increase MMP reduction and also decrease caspase-9 activity induced by MPP^+. F4d1 and F4c4 could not inhibit MMP declining and caspase-9 activity enhancement suggesting that mitochondrial pathway is not involved in the neural protective effect of these fractions. Increasing evidence has demonstrated that inflammation is the fundamental process contributing to DA neurodegeneration in PD.[31] It has been reported that acacetin, a flavonoid compound, can protect DA neurons against the neurotoxicity involved in PD through inhibiting the production of inflammatory factors, including nitric oxide, prostaglandin E2 (PGE2), and COX-2. Pro-inflammatory cytokines such as interleukin (IL)-1, IL-8, and tumor necrosis factor-α can stimulate COX-2 that leads to overproduction of PGE2.[32] It has been shown that acacetin protects dopaminergic cells against MPP^+-induced neuroinflammation *in vitro* and *in vivo*.[33] It is also reported that FAs have anti-inflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases.[34] FA can inhibit COX and 5-lipoxygenase pathways related to arachidonate metabolism for the production of eicosanoids in membrane lipid peroxidation in the peritoneal leukocytes of rats.[34,35] Therefore, to further explore the molecular mechanisms that mediated the enhancement of cell viability, we evaluated the effect of F4d1, F4c4, and F4c5 on COX activity in PC12 cells. MPP^+ sharply increased COX activation, but FA mixtures pretreatment significantly decreased MPP^+-induced COX activity. Based on these results, in conclusion, our results provided novel insights into the cellular mechanisms of FA's biological functions. The anti-inflammatory activity of *N. sativa* L. seeds has demonstrated in before studies.[36] In one study, anti-inflammatory effect of *N. sativa* L. seeds has been examined using carrageenan-induced edema in rat hind paws and cotton seed pellet in rats. A significant dose-dependent anti-inflammatory effect was shown, and it was suggested that the anti-inflammatory activity of the *N. sativa* seeds may be due to inhibiting the generation of eicosanoids and lipid peroxidation.[37]

Neuroprotection of FA mixture composed of LA, OA, and PA may be associated with alleviating apoptotic pathway. Furthermore, it is possible that the anti-inflammatory property of FA mixture contributes to their neuroprotective effects against MPP^+-induced cell injury. These results suggest that FA mixture isolated from *N. sativa* might be a promising candidate for the prevention or treatment of neurodegenerative diseases such as PD, but further studies to understand the basic mechanism are required.

CONCLUSION

Bioassay-guided fractionation of *N. sativa* seed extract resulted in the isolation of FA mixture of LA, OA, and PA, which decreased the COX activity induced by MPP^+ in PC12 cells in subtoxic concentration. They may exert cytoprotective effects through their anti-apoptotic and anti- inflammation actions and could be regarded as a dietary supplement.

Financial support and sponsorship

Financial supports of Vice Chancellery for Research and Technology of Kermanshah University of Medical Sciences is acknowledged specially for grant No. 94267. This article has been extracted from Pharm D Thesis of Hoda Monaghash, Major of Pharmacy, Kermanshah University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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