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Research Article

Fatty Acid Mixtures from *Nigella sativa* Protects PC12 Cells from Oxidative Stress and Apoptosis Induced by Doxorubicin

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ABSTRACT

Background: Fatty acids (FAs), the key structural elements of dietary lipids, are notable in the nutritional value of plants. Black cumin, a popular anti-inflammatory and antioxidant food seasoning, contains nonpolar constituents such as FAs.
Methods: Seeds were extracted using hexane and their cytoprotective activity was assessed against doxorubicin (DOX)-mediated oxidative stress and apoptosis in PC12 cell line.
Results: In spite of the cellular death induced by DOX toward PC12 cells, bioassay-guided purification showed that pretreatment with FAs mixtures (24h) attenuated DOX-mediated apoptosis, which could be attributed to the inhibited caspase 3 activity and enhanced mitochondrial membrane potential. Palmitic acid, caprylic acid and oleic acid each 1/3 in the mixture, also suppressed DOX-induced ROS generation.
Conclusion: Our observation indicated that the subtoxic concentration of FAs from *Nigella sativa* could effectively protect the cells against oxidative stress, due to their antioxidant activity, and could be regarded as a dietary supplement.

Introduction

Nigella Sativa L. (Ranunculaceae), black cumin, is a popular medicinal plant and food additive in Western and Southern Asia. It is traditionally used as lactogogue, carminitative and antihelmenthic, muscle relaxant, antifebrile and as immunity modulator agent.[1,2] Besides, several pharmacological and biological experiments have been performed on the plant confirmed its vast medicinal use.[3,4]

As a natural antioxidant, *N. sativa* could exert various effects. It is added to bread and rice to prevent GI inflammation and flatulence. Traditionally, it is believed that *N. sativa* oil would prevent and cure inflammatory chronic liver, spleen, kidney, respiratory and rheumatoid ailments[1,2,5] which have been recently proved *in vitro* or *in vivo*. For instance, black cumin constituents possessed the activity to prevent the hypertention induced by l-NAME and treat the renal damage by decreasing the elevated creatinine and increasing GSH levels in rats.[6] *N. sativa* showed gastroprotective effects against alcohol[7] and ischemia/reperfusion[8] both in rats and colon protection induced by acetic acid in rats as well.[9] Black cumin phytochemicals were scavengers of various reactive oxygen species[10] and could successfully inhibited the carcinogenesis via regulating lipid peroxidation and cellular antioxidant milieu.[11,12]

Regarding neuroprotectivity, *N. sativa* extracts and constituents exerted protective effects against transient forebrain ischemia-induced neuronal damage[13] and chronic toluene exposure[14] both in the rat hippocampus. Also, protective effects in sciatic nerves in Streptozotocin-induced diabetic rats with decreasing Myelin breakdown[15] protective effects in Parkinson's disease[16] have been documented.

Several fatty acid (FA) rich plants show promising antioxidant effects[17, 18] and antioxidant activity of non-polar extracts were positively correlated with polyunsaturated Fas.[19] In order to find new neuroprotective components from *N. sativa*, in the current study we focus on the FA antioxidant effect rather than that of thymoquinone, a popular bioactive constituent of black seeds.

Doxorubicin, an anthracycline antibiotic, is a highly effective antineoplastic agent against malignant lymphoma, acute leukemia and a wide range of solid tumors. However, adverse toxic effects related to the dose of this drug including neurotoxicity which could be resulted in neuro-degeneration diseases and limit doxorubicin use.[20] Doxorubicin was found potent in induction of oxidative stress and apoptosis towardPC12 cells which makes it appropriate to be used within *in vitro* models for assessment of neural cell death.[21] The present study aims to investigate the protective effect of compounds isolated from the hexane extract of the seeds of *Nigella sativa* on neural cell death induced by doxorubicin in PC12 cells.

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Materials and Methods

General instruments and chemicals

NMR spectra were obtained using a Bruker® (400 MHz) spectrometer Mass spectrum was measured using an Agilent 5973 Network mass selective spectrometer. Silica gel and HPLC Eurospher II (Normal phase, Si 250×20 mm columns were utilized for purification of the samples MTT powder (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) and cell culture medium were procured from Sigma Aldrich (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively. Hydrogen peroxide H_2O_2, Fatty acid standards, and 2,5dichlorofluorescindiacetate (DCF-DA) were supplied from Sigma. penicillin-streptomycin, and fetal bovine serum (FBS) were procured from Gibco. All of the solvents performed in this study were supplied from Dr. Mojallali (Iran).

Plant material

Seeds of *Nigella sativa* L. were bought from Faculty of Agriculture, Razi University of Medical Sciences. Then, seeds were planted in appropriate condition and plant material was dried and pressed. Herbarium specimen was identified by Dr. NastaranJalilian, Department of Biology, Razi University, Kermanshah (No. 248008007).

Bioguided fractionation and purification of active constituents

Black cumin seeds were hot extracted with hexane and defatted using methanol and freezing. Cold methanol soluble portion was fractionated using silica gel and hexane ethyl acetate mixture to get 5 fractions as F1 to F5. Fraction F4, which was the most active fraction in biological tests as will be explained in results section, was further purified using another normal phase column with Hexane in EtOAc (80% to 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 to F4d3, the other F4c1 to F4c5.

To identify structures samples were sent for spectroscopic analyses.

Cell culture and treatment

PC12 cells, rat pheochromocytoma-derived cells , were cultured in a medium supplemented with fetal bovine serum (10% v/v) and 1% penicillin/streptomycin and L-glutamine and were kept in an incubator with 37 °C and 5% CO2.To assay of protective effect of Fatty acid mixtures from Nigella sativa on apoptosis and DOX –mediated oxidative stress in PC12 cells, the experiments were conducted on four different groups of cells; control cells (which have not been treated by fractions and DOX), fraction-treated cells (treated for 24 h with different doses of fractions(0-50 µg/cc) and sub-fractions (0-300 µg/cc))DOX-treated cells (treated for 24 h with DOX (0-45 µM)) , and fraction-pretreated DOX-treated cells(pretreated with non-toxic concentrations of fractions and sub-fractions and then for 24 h, cells were treated with IC_{50} concentration of DOX (5.8 µM) for another 24 h).

Viability assay

MTT assay was performed to examine the cytotoxic activity of DOX, fractions and sub-fractions toward PC12 cells.[22] Cells were seeded in a 96-well culture plate. In order to prepare the stock solutions, all the compounds were dissolved in dimethyl sulfoxide (DMSO), while the concentration of DMSO in the culture medium kept constant at 0.5% for all the solutions. At the predetermined time intervals, the culture medium was replenished by 200 µl of MTT(0.5 mg/ml) in growth medium and then the plates incubated at 37 °C for 3 hr. After the incubation period, and to solubilize the formazan crystals, 200 µL DMSO was added to each well. The absorbance was determined on an ELISA plate reader (Biotek, H1M) with a test wave length of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630).

Intracellular ROS assay

The levels of intracellular ROS were examined by a fluorometry assay using DCF-DA, as a cell permeable dye -which could passes through the plasma membrane into the cytosol, where the acetate group is rapidly removed by non-specific esterases.[22] PC12 cells were cultured at 6 well plate (1×10^6). At appropriate time interval, 2′,7′-dichlorofluorescein diacetate (Sigma- Aldrich Germany)(20 µM) DCF-DA were added to the culture medium of cells and after half an hour of incubation at 37° C, the cells are washed with cold PBS. Then, cells are suspended in PBS solution containing 10 mM EDTA and the fluorescence intensity which represents the amount of ROS was measured at the excitation wavelength of 485 nm and emission wavelength of 530 nm, respectively, by fluorescence microplate reader.

Caspase 3 activation assay

To investigate the activity of caspase-3 enzyme, PC12 cells were cultured at 6 well plate (1×10^6). After treatment, cells were collected and lysed using cold lysis buffer and were kept on ice for ten minutes. Afterwards, cell lysate was centrifuged for five minutes at a temperature of 4°C and before adding the substrate of caspase -3 (Ac-DEVD-pNA) (Sigma America).[21] To measure the caspase 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 red color. After adding the substrate of caspase -3 (Ac-DEVD-pNA), it was incubated for one h at 37 °C and then, light absorption of pNA was measured at a wavelength of 400- 405 nm using spectrophotometer. Comparing pNA absorbance of the apoptotic sample with control sample indicates the rate of caspase activity.

Mitochondrial membrane potential assay

To evaluate the mitochondrial membrane potential, PC12 cells were cultured at 6 well plate (1×10^6). After

treatment the fluorescence color of Rhodamine 123 with a concentration of 20 mM was added to the cells and cells were incubated for further 30 min at 37°C. Then, the cells were using PBS solution and the amount of their fluorescence was determined at a wavelength of 488-510 nm using fluorescence microplate reader.[23]

Statistical analysis

Prism software is used for analyzing the data related to protective effects and IC_{50} 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 post-test are used.

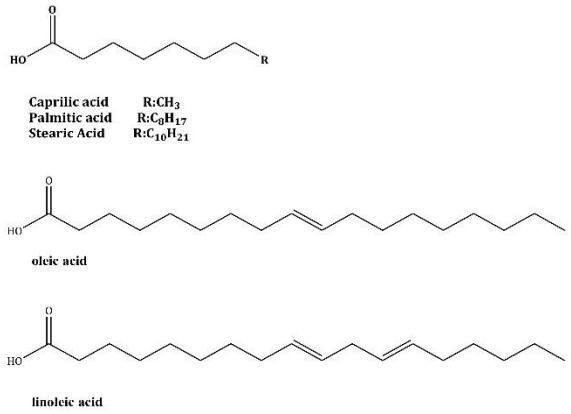

Figure 1. Structure of bioactive fatty acids isolated from *Nigella sativa* seeds.

Results

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.[24-27] F4d1 to F4d3 and F4c1 to F4c5 determined to be fatty acid derivatives in different ratios. Active fractions composition is presented in Table 1 and structure of fatty acids are in Figure 1.

Linoleic acid(LNA); ^{1}HNMR ($CDCl_3$, 400 MHz) in Table 2.EI MS: m/z 280 $[M]^+$, 206 $[M-74]^+$, 164 $[M-116]^+$, 249 $[M-31]^+$, m/z =74.

Oleic acid(OLA): ^{1}HNMR ($CDCl_3$, 400 MHz)in Table 2. EI MS: m/z 282 $[M]^+$, 208 $[M-74]^+$, 166 $[M-116]^+$, 250 $[M-32]^+$, m/z =74.

Palmitic acid(PAM): ^{1}HNMR ($CDCl_3$, 400 MHz) in Table 2. 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.

Caprylic acid: ^{1}HNMR ($CDCl_3$, 400 MHz) in Table 2. EI MS: m/z 144 $[M]^+$, 70 $[M-74]^+$, 101 $[M-43]^+$, 113 $[M-31]^+$, m/z = 87, 101, 115, 129, 143, 157, 199, etc. of general formula $[CH_3OCO(CH_2)_n]^+$, m/z =74.

Stearic acid: ^{1}HNMR (CDCl3, 400 MHz) in Table 2. EI MS: *m/z* 284 $[M]^+$, 60 (Mc Lafferty rearrangement ion) $[M-196]^+$, 73 $[M-211]^+$, 267 ($[M-17]^+$), 115 to 255 $[HOOC(CH_2)_n]^+$.

Based on HNMR data, the active fractions contain fatty acid derivatives. A peak at δ 5 to 6, representing unsaturated FA, along with other common features like triplet at *ca.* δ 0.9 (terminal CH3), hump of hydrogen resonance at δ 1.5-2 (homologue CH2 series (CH2)n), and several peaks at δ 1.7-2.8 related to more deshielded methylenes. According to retention time of compounds in GC column compared to those of fatty acid 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 for oleic acid, and homologue ions $[55 + CH_2)n]^+$ are abundant. The Mc Lafferty 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 $[M-17]^+$, Mc Lafferty rearrangement ion at *m/z* 60 and 73, and homologue fragments obtained from methylene lose from $[HOOC(CH2)n]^+$ ranged *m/z* 115 to 255.

Table 1. Fatty acid composition of bioactive fractions.

	Palmitic acid	Caprylic acid	Oleic acid	Linoleic acid	Stearic acid
F4d2	45%	30%	25%	-	-
F4d3	13%	-	21%	34%	-
F4c2	10%	-	11%	34%	14%

*The composition is determined by GC analysis using standards.

Table 2. ^{1}HNMR data of isolated fatty acids (400 MHz, CDCl3).

	Linoleic acid	Oleic acid	Palmitic acid	Caprylic acid	Stearic acid
H2	2.38, m*	2.38, t	2.38, t	2.38, t	2.38, t
H3	1.67, s	1.64, m	-	-	1.34, m
H4,5,6	1.22, d	1.34, m	1.34, m	1.22, d	1.34, m
H7	1.35, d	1.34, m	1.34, m	1.35, d	1.34, m
H8	2.08, s	2.02, m	1.34, m	0.92, t	1.34, m
H9, 10	5.40, m	5.39, t	1.34, m	-	1.34, m
H11	2.81, t	2.02, m	1.34, m	-	1.34, m
H12,13	5.40, m	1.34, m	1.34, m	-	1.34, m
H14	2.08, s	1.34, m	1.34, m	-	1.34, m
H15	1.35, d	1.34, m	1.41, t	-	1.34, m
H16	1.22, d	1.34, m	1.34, m	-	1.34, m
H17	-	-	-	-	1.34, m
H18	-	-	-	-	0.92, t

Chemical shift in ppm, multiplicity.

Effect of fractions Pretreatment on DOX-Induced Cell Death

The cytotoxic activity of different concentrations of DOX against PC12 cells were assessed (IC50=5.8 µM). As shown in Figure 2a, DOX induced cytotoxicity in a concentration dependent manner. Our MTT results showed that the extract of *N. sativa* at the non-toxic concentration (12.5 µg/cc) increased significantly the viability of PC12 cell line (Figure 2b, c).

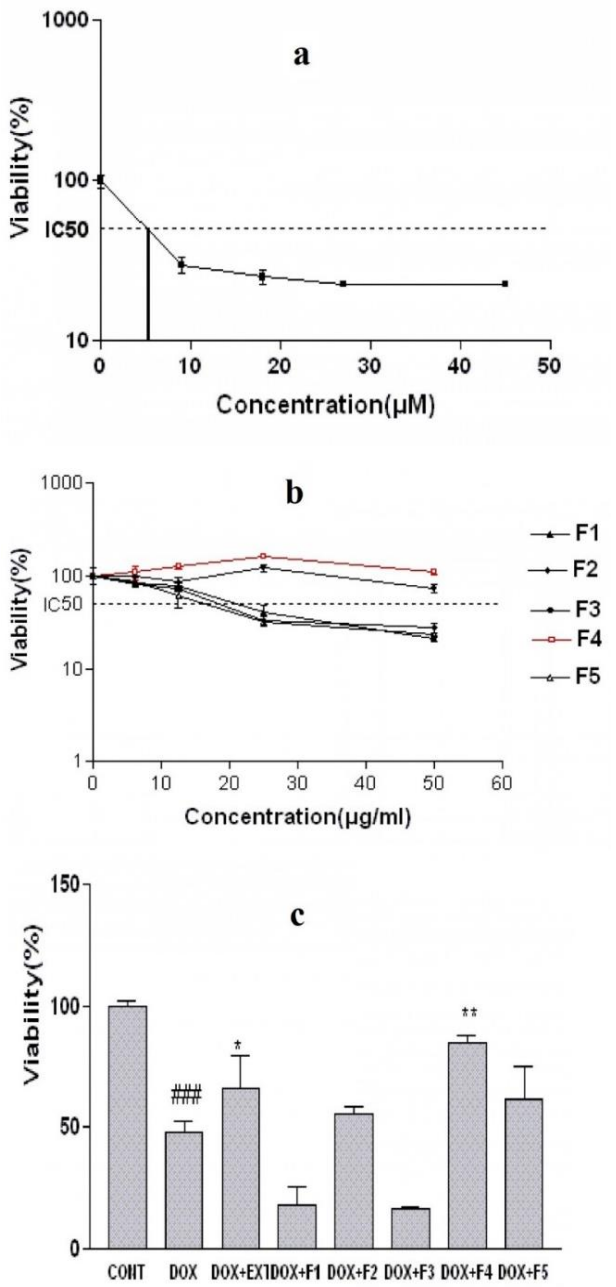

Figure 2. Effect of *Nigella sativa* constituents pretreatment on DOX-induced cell death in PC12 cells. **a)** The effect of 24 h treatment with DOX (0-45 µM) on the viability of the cells. **b)** The effect of 24 h treatment of hexane extract (EXT) and fractions (F1-F5) (0-50 µg/ml) alone on the viability of the cells. **c)** The effect of EXT and F1-F5 (0-50 µg/ml) on cytotoxicity induced by 24 h treatment with IC_{50} concentration of DOX (5.8 µM) in PC12 cells.The cell viability was determined by MTT assay as described in materials and methods. Data are expressed as the mean ± SEM of three separate experiments.

In the next set of our experiment, in order to find the non-toxic concentrations of fractions (0-50 µg/cc) which could potentially inhibit the cytotoxicity mediated by DOX, the effects of treatment with different doses of fractions on the viability percentage of PC12 cells was also examined. The results are shown in Figure 2b.

The figure clearly revealed that the hexane extract and fractions F1, F2, and F5 caused no significant effect on the viability of the cells at concentrations up to 20 µg/cc. Fractions F3 and F4 induced nono significant toxic effect against PC12 cells up to 50 µg/ml (relative MTT activity >80%).Using the non-toxic concentrations of fractions, another experiment was performed to elucidate the effects of exposure of these fractions on the viability of cells pretreated with DOX. Among fractions, fraction F4 (25 µg/ml) was found the most potent fractionagainst DOX-induced toxicity in PC12 cells. Based on this information, fraction F4 was further purified in three steps to get eight subfractions, F4d1 to F4d3 and F4c2 to F4c5. As shown in the Figure 3 except for F4c2, all subfractions possessed no cytotoxic effect toward PC12 cells at concntration up to 200 µg/ml. when the effect of nontoxic concentrations of subfractions was evaluated on cell injury induced by DOX, our results showed that cytotoxic effects of DOX on PC12 cells were significantly blocked by pretreatment with F4d2, F4d3, F4c2 (Figure 3b). Therefore, in subsequent experiments, these subfractions were selected for identification of mechanisms of cytoprotective action in the PC12 cell line.

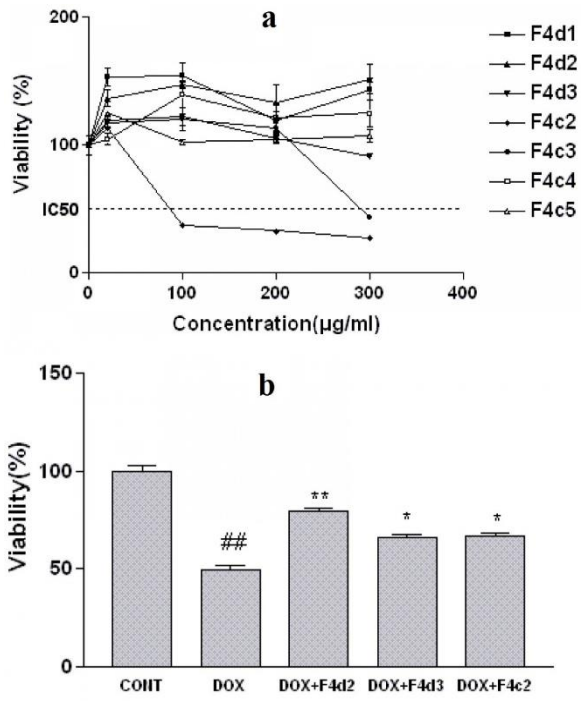

Figure 3. a) The effects of 24 h treatment with fatty acid mixtures, F4d2, F4d3 and F4c2 (0-300 µg/ml) on the viability of cells. b) The protective effects of F4d2, F4d3 and F4c2 (6.25 µg/ml) on DOX-induced cytotoxicity (5.8 µM). The cell viability was determined by MTT assay as described in materials and methods. Data are expressed as the mean ± SEM of three separate experiments.

Effects of N. sativa fractions on Oxidative Stress Induced by DOX

The DOX-induced ROS generation was assessed to undrestant whether DOX can significantly mediate oxidative stress in cells. As anticipated, DOX-treated cells showed higher ROS level, rather than control group.. The protective effect of fractions on DOX-induced ROS generation wasalso examined . As it can be observed in Figure 4, the ROS level was found considerably lower after exposoure of F4d3, F4d2 and F4c2 confirming the protective effect of these samples against DOX-induced ROS production.

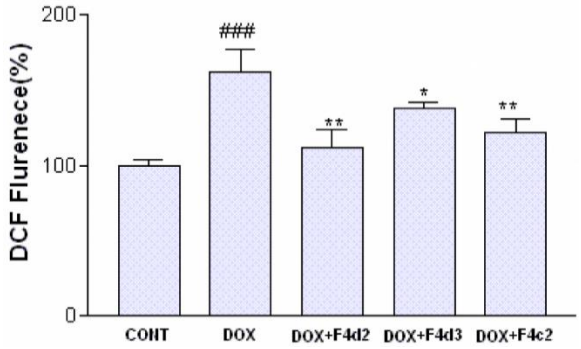

Figure 4. Effect of fatty acid mixtures from Nigella sativa (6.25 µg/ml) on intracellular formation of reactive oxygen species (ROS)triggered by exposure of PC12 cells to DOX(5.8 µM) in the pre-incubation experiments assessed by DCF assays. Data are expressed as the mean ± S.E.M of three separate experiments. ### p<0.001 vs. Control, * p<0.05, **p<0.01 vs. DOX treated cells.

Effect of fatty acid mixtures on Mitochondrial Membrane Depolarization (MMP) induced by DOX

In order to elucidate how DOX and/or selected sub-fractions can alter the mitochondrial events, the collapse of MMP in the cell line was examined with a cell permeable cationic fluorescent dye, Rhodamine 123. A gradual decrease in the fluorescence intensity was observed after exposure of IC_{50} concentration of DOX for 24 h ($P < 0.05$). All sub-fractions attenuated the MMP changes in DOX treated PC12 cells. The increase in fluorescence intensity was observed 43%, 25% and 34% for cells pretreated with F4d3, F4c2 and F4d2, in comparison to DOX-treated group (51%). (Figure 5)

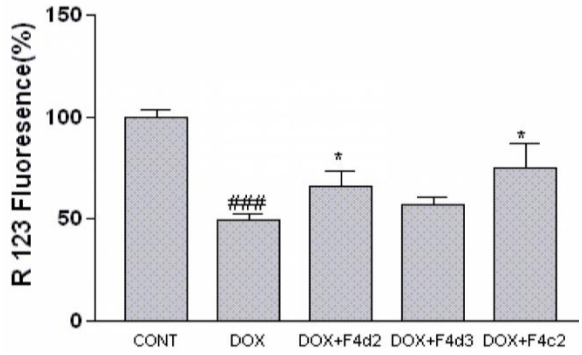

Figure 5. Effect of fatty acid mixtures (6.25 µg/ml) from*Nigella sativa* on DOX (5.8 µM) -induced mitochondrial membrane potential (MMP) collapse as detected by Rhodamine 123. Data are expressed as the mean ± SEM of three separate experiments. ###p<0.001 vs. control, *p<0.05, *p<0.05 *vs.* DOX treated cells.

Effect of fatty acid mixture Pretreatment on Caspase-3 Activation

The caspase-3 activity is known as one of the most important key apoptosis-mediators in mammalian.[28] DOX-treated cells exhibited 93% increase in caspase-3 activity rather than un-treated cells. In contrast, F4d3 and F4c2-treated groups possessed lower caspase-3 activity in comparison to the DOX-treated ones (Figure 6). These observations corroborate the effect of F4d3 and F4c2 in the inhibition of DOX induced caspase-3 activity.

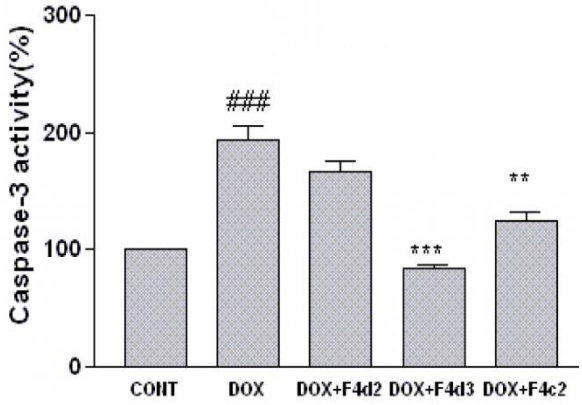

Figure 6. Effect of fatty acid mixtures from *Nigella sativa*(6.25 µg/ml)on caspase-3 activity. Cells pretreated with mixtures 24 h before exposure to IC_{50} concentration of DOX (5.8 µM). Caspase-3 activity was measured by colorimetric detection of p-nitroaniline and expressed as percent of control. Data are expressed as the mean ± S.E.M. of three separate experiments. ### p<0.001 *vs.* Control,*** p<0.001, **p<0.01 vs. DOX treated cells.

Discussion

We have previously reported that DOX, a widely anticancer drug, induces oxidative stress and apoptosis in PC12 cells.[29] The aim of this study was to test a series of fatty acids, including oleic acid, palmitic acid, caprylic acid and linoleic acid, isolated from the hexane extract of *N. sativa* seeds, to assess their protective effect against DOX-induced neuronal damage. Our results showed that subfractions F4d3, F4d2 and F4c2 exhibited a potent protective effect DOX-induced cell injury. Therefore, to further elucidate the mechanisms by which these compounds protect PC12 cells from DOC-induced cell death, we examined key parameters involved in apoptosis. The mitochondria play a crucial role in activating apoptosis in mammalian cells. Mitochondrial membrane permeabilization leads bioenergetics failure and permits the release of soluble molecules from the outer space of the mitochondria to the cytosol. This ultimately leads to cytochrome c release and caspase-9 activation.[30] Downstream caspases such as Caspase-3 and caspase-9, are activated by mature caspase-9,in turn for the execution of cell apoptosis. In turn, caspase-3 activates downstream caspases in a proteolytic cascade.[31] As anticipated, in the present study, a decrease in MMP and an increase in caspase-3 activation were observed after treating PC12 cells with DOX. It was observed that exposure of PC12 cells to DOX leads an increment in the ROS level, which was in consistence with the results of our previous studies. Moreover, we showed that F4d2 and F4c2 are able to

increase the DOX-induced MMP reduction. It seems that the mitochondrial pathway of apoptosis was not involved in the neural protective effect of F4d3 because the MMP did not increase after exposure to F4d3 in PC12 cells. It must also be noted that pretreatment with F4d2 increased the MMP but did not attenuate caspase-3 activation. As noted earlier, upon permeabilizing mitochondrial outer membrane cytochrome c releases from the mitochondrial intermembrane space (IMS). In addition, apoptosis-inducing factor (AIF) can be released from the mitochondrial outer membrane.[32]

Many studies have demonstrated that AIF is a caspase-independent cell death mediator[33] and can actively induce CICD following the mitochondrial release. AIF translocate to the nucleus and then mediates chromatin condensation and caspase-independent cell death.[34] It is possible that F4d2 protects PC12 cells through the inhibition of AIF release. Mechanistically, F4d2, F4d3, and F4c2 significantly inhibited ROS-generation in DOX-treated PC12 cells. Hence, According to the results of this study, the neuroprotective activity of F4d2, F4d3 and F4c2 could be attributed to their antioxidant or radical scavenging effects. Studies have identified the protective role of saturated and unsaturated FAs in biological systems. Perdomo et al. evaluated the cardio protective effect of oleic acid against cardiovascular insulin resistance in myocytes.[35] They showed that oleate is protective, prompts the endothelial dysfunction to respond the pro-inflammatory signals, and reduces proliferation and apoptosis in myocytes. In another study, it was investigated whether oleic acid can inhibit the palmitic acid-induced apoptosis toward pancreatic cells (AR42J).[36] The results indicated that the restoration of the anti-apoptotic/pro-apoptotic protein balance from apoptosis toward cell survival is involved in the cytoprotective effects of oleic acid against PAM-induced apoptosis in pancreatic AR42J cells. However, there has been no previous suggestion of the neuronal cell protecting activity of oleic acid for treating or inhibiting neuronal injury till now. It must be noted that relationship between FAs consumption and antioxidant effects is a matter of challenge and controversial. The antioxidant effects of olive oil are probably due to its high oleic acid content. Although several experiments have shown the beneficial effects of FAs use in preventing oxidant-related diseases.[37] Rizos et al[38] reported that administration of omega-3 PUFA could not cause decreasing the risk of all-cause mortality, myocardial infarction and cardiac death, sudden death, , or stroke.

Conclusion

We conclude that F4d2, F4d3, and F4c2 have protective effects against DOX-induced cytotoxicity in the PC12 cells. These effects possibly occur through their antioxidant activity and antiapoptotic properties. Accordingly, it is more likely that among the lipid components, linoleic acid and palmitic acid are responsible for the protective effect of F4d2, F4d3, and F4c2 in prevention of oxidative stress and apoptosis mediated by DOX toward the PC12 cells. Finally, the use of black cumin oil and non-polar fractions could be proposed as a protective and antioxidant supplement similarly as some other FA-containing plants such as evening primrose[39] are used.

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Conflict of interests

The authors claim that there is no conflict of interest.

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