



Research Paper

Neuroprotective and antioxidant activity of *Nigella glandulifera* Freyn & Sint seeds

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ABSTRACT

In many neurodegenerative diseases, the excess levels of reactive oxygen species (ROS), produced by oxidative insults, leads to cellular damage and apoptosis. Therefore, the development of nontoxic and highly bioactive antioxidant compounds is important to prevent generation of free radicals. In recent years, naturally occurring bioresources have been received close attention for their potential role in regulating the pathways involved in oxidative stress and apoptosis. *Nigella sativa* L seeds (NS) and *Nigella glandulifera* Freyn & Sint seeds (NG) have been considered as promising bioactive-rich sources of antioxidants that could potentially prevent oxidative stress-induced neurodegenerative diseases. Thus, the present study investigated the neuroprotective effect of volatile oils of NS and NG against hydrogen peroxide H₂O₂-induced neurotoxicity in PC-12 cells. The volatile oils of NS and NG protected PC-12 cells against H₂O₂-induced neurotoxicity through preserving the mitochondrial metabolic enzyme activities, and regulating anti- and pro-apoptotic gene expressions, thus reducing apoptosis. These findings suggest that the volatile oils of NS and NG may be an effective antioxidant that could prevent oxidative stress-induced neurodegenerative disorders.

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INTRODUCTION

Nigella is one of the key recipes of many highly valued and frequently used folk medicines including Mongolian medicine, Uyghur medicine and Chinese medicine. The genus of *Nigella* includes 18 species of annual plants. Among them, *Nigella sativa* is the most valued species and has records of usage in herb medicines in the Middle East area for more than 3000 years (Yun et al., 2014; Gharby et al., 2015; Suh et al., 2013). "Flora of China" records another species of *Nigella*, *Nigella glandulifera* Freyn & Sint., which is cultivated as a medicinal plant in areas such as Yunnan province, Guangxi and Xinjiang Autonomous. *Nigella* is one of the key components of several widely used Mongolian herbal formulations. For example, Eerdun Wurile (Gaowa et al., 2018), a traditional Mongolian medicine with clinically proven neuroprotective activities, contains the seed of *N. glandulifera* Freyn & Sint. The active component of *Nigella*,

thymoquinone (TQ) (Gharby et al., 2015; Ali and Blunden, 2003), is a bioactive molecule with remarkable anti-oxidative activity. In rat model of arthritis, TQ effectively increased the levels of GSH, CAT and SOD while significantly reducing the levels of pro-inflammatory mediators such IL-1b, IL-6 and TNF-a (Umar et al., 2012; Ahmad et al., 2013).

Oxidative stress is commonly implicated in the pathogenesis and progression of a wide range of diseases including neurodegenerative diseases (Mayne, 2003; Smith et al., 1996). Reactive oxygen species (ROS) induces oxidative stress, which leads to cellular damage, mitochondrial dysfunction and apoptosis (Ismail et al., 2012; Kadowaki et al., 2005; Lin et al., 2015; Heo et al., 2009). These processes are counteracted by antioxidant defense systems, which scavenge ROS by normalizing their levels beyond physiologically acceptable limits (Miller and

Sadeh, 2014). Even though, cellular damage occurs when ROS level passes a certain threshold, resulting in lipid peroxidation, protein inactivation, as well as DNA damage (Ismail et al., 2014; Bak et al., 2014). The neuronal oxidative stress plays important roles in neurodegenerative diseases (Ismail et al., 2014). Antioxidants reduce neuronal degeneration by developing antioxidant systems, which prevent the excess production of free radicals (Zhang et al., 2008). The antioxidant systems include antioxidant enzymes, such as catalase (CAT), which converts H₂O₂ into molecular oxygen and water; superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anions to hydrogen peroxide; glutathione peroxidase (GPx), which catalyzes the degradation of H₂O₂ and hydroperoxides (Vertuani et al., 2004).

Although *N. glandulifera* Freyn & Sint. has been used in many folk medicine in China, its chemical components, especially the TQ content and the anti-oxidative activities in neural cells has never been studied in details (Besma et al., 2013). In this study, we identify the species of *Nigella* by DNA sequencing, analyze the TQ content by NMR based novel method, and measured anti-oxidant activities in PC-12 cells by comparing the two *Nigella* species.

MATERIALS AND METHODS

Reagents

N. sativa L. seeds (NS) and *N. glandulifera* Freyn & Sint seeds (NG) were purchased from North Park Produce (Poway, CA, USA) and Yishengtang (Guangxi, China), respectively. Thymoquinone (TQ) was purchased from Sigma-Aldrich, USA. Superoxide Dismutase (SOD), catalase (CAT) and reactive oxygen species (ROS) were purchased from NjjcBio (Nanjing, China). Calcein-AM/PI Double Stain Kit and MTT were purchased from Yeasen, China and Keygen Biotech, China, respectively.

Extraction of volatile oils from NS and NG.

The volatile oils of NS and NG were extracted by petroleum ether. Briefly, 500 mg of the seeds were grinded in powder and placed into extraction device in 50 mL of petroleum ether. The mixture was stirred at 80°C for 8 h and followed by filtration, rotary distillation, nitrogen drying and then calculated yields of the extracts. The volume of the TQ in extracted volatile oils was determined by proton nuclear magnetic resonance (¹H-NMR) spectrometer.

Measurement of TQ content by proton NMR

A stock solution of standard TQ (10 mg/mL) was prepared in chloroform. Different volumes of working TQ solution were prepared to build calibration curve in 0.0625, 0.125,

0.25, and 0.5 mg/ml and the solutions were analyzed by ¹H-NMR.

Cellular toxicity

PC-12 cells were cultured in a 96-well plate at a density of 1×10⁴ in RPMI 1640 medium (BI, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (100 U/mL penicillin 100 µg/mL streptomycin) (Gibco, USA) and incubated for 24 h at 37°C in 5% CO₂. After NS and NG were added into the cell culture at final concentration of 25, 50, 80, 120 and 150 µg/mL, the cells were incubated for additional 24 h. The cell viability was measured by MTT assay. For neuroprotective effect of NS and NG on PC-12 cells, after the cells were incubated with various concentration of NS and NG for 24 h, the cells were challenged with 250 µM of H₂O₂ for more 4 h. Then, MTT assay was carried out for detection of cell viability.

Assays for antioxidant enzymes

The cells were seeded in 6-well plates at a rate of 6×10⁵ cells/well. After 24 h of incubation, the cells were treated with TQ (positive control; 1 µg/mL), or different doses of NS and NG (80 or 120 µg/mL) for 24 h. After the cells were challenged with 250 µM of H₂O₂ for 4 h, the cellular protein was extracted using M-per reagent (Thermo, USA). Protein concentration was determined using BCA Protein Assay Kit (Thermo, USA). To measure the cellular antioxidant enzymes, CAT and SOD, the extracted protein was analyzed using CAT and SOD detection kit (NjjcBio, China) according to the manufacturer's instructions.

Intracellular ROS assay

Generation of ROS was evaluated using the 2,7-dichlorofluorescein diacetate (DCFH-DA) method. Briefly, PC-12 cells were seeded at a density of 2 × 10⁴ cells/mL in a black 96-well plate. Prior to treatment, cells were incubated with 10 µM DCFH-DA (dissolved in DMSO) for 30 min in the dark at 37°C and washed twice with PBS. The cells were then pretreated with TQ (1 µg/mL) and different concentration of NS and NG (80 or 120 µg/mL) for 24 h and subsequently challenged with 250 µM H₂O₂ and subjected to fluorescence measurement by excitation at 485 nm and emission at 535 nm using a microplate reader (FilterMax F5, USA). The fluorescence intensity is proportional to the intracellular ROS levels.

Live/dead assay on PC-12 cells

PC-12 cells were cultured in 12 well plate at a rate of 3×10⁵

cells/well for 24 h and treated with TQ (positive control; 1 $\mu\text{g}/\text{mL}$), NS (120 $\mu\text{g}/\text{mL}$) and NG (120 $\mu\text{g}/\text{mL}$) for more 24 h. After 250 μM of H_2O_2 was applied to the cells for 4 h, calcein AM/propidium iodide (PI) assay was assessed following the manufacturer's protocol for detecting live/death cells.

Statistical analysis

Statistical analysis was conducted by one-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons test. $P < 0.05$ was considered as significantly different. The analysis was performed using GraphPad Prism 7.0.

RESULTS AND DISCUSSION

Identification of *Nigella* seeds by DNA sequencing

Accurate identification of medicinal plant is crucial for the precise and effective clinical application. Morphological observation and phytochemical analysis can provide substantial information for the identification of the plants. However, such methods have apparent limitations such as damage to the morphology as well as significant changes to the chemical compositions due to the inappropriate storage and preservation conditions. DNA barcoding based on the highly preserved genomic sequences such as internal transcribed spacer (ITS), *rbcL* and *matK* can provide accurate identification of plant species. To identify the two *Nigella* sp., we performed ITS sequencing of genomic DNA extracted from the seeds of *N. sativa* and *N. glandulifera* Freyn & Sint. (Figure 1).

Sequencing of a 855 nt ITS region of the two species showed a 11.9% difference, indicating that *N. sativa* and *N. glandulifera* Freyn & Sint. are two distinct species, which may have diverse secondary metabolites and bioactive chemical components.

Quantification of thymoquinone (TQ) content of *Nigellas* by ^1H NMR

NMR is a powerful quantitative analytical tool. Because the integrated intensity of a resonance signal is proportional to the number of nuclei represented by that signal, all protons across the spectrum are equally sensitive, therefore compound-specific extinction coefficients or calibrations is usually required for determination of quantitative results. Quantitative NMR (qNMR) has been shown to be particularly useful in analysis of bioactive natural product. While the content of TQ in *N. sativa* has been studied, quantification of TQ content in *N. glandulifera* Freyn & Sint. seeds is rarely reported. Quantification of natural compounds can be achieved by chromatographic separation

and subsequent spectroscopic detection (e.g., UV/Visible) or spectrometric detection (e.g., mass spectrometry). Our first attempt for quantification of TQ in black seed using UPLC-qTOF technique failed due to the very low response factors of TQ (data not shown). Therefore, we use ^1H NMR to determine the content of TQ in *Nigellas* (Figure 2).

To quantify TQ in NS and NG oil extracts, proton nuclear magnetic resonance (^1H -NMR) spectrometer was performed. Firstly, the calibration curve was found to be linear with TQ concentration of 0.0625, 0.125, 0.25 and 0.5 mg/mL. The linear regression equation of the peak area and its mass ratio was $Y=0.493x-0.0005$, where Y is response and x is the amount of TQ and the correlation coefficient (R^2) was 0.99942 which was highly significant ($P<0.05$). Then, the intensity of TQ absorption peaks of NS and NG oil extracts, which was analyzed by ^1H -NMR, was calculated (Table 1) to estimate TQ contents by comparing with the standard curve under the identical conditions (Figure 1). As the results, TQ content of NS and NG was 0.153 and 0.224 mg/mL, respectively.

The cytotoxic effects of NS and NG on PC-12 cells

The cell survival rate of NS and NG treated PC-12 cells were evaluated in the absence or presence of H_2O_2 by MTT assay. The percentage of viable cells was determined comparing them with the non-treated cells (NT). Treatment of PC-12 cells with NS or NG (25–150 $\mu\text{g}/\text{mL}$) did not show significant cytotoxicity (Figure 3A). The neuroprotective effects of NS and NG were then determined by pretreating the cells with NS and NG for 24 h and subsequently exposed to 250 μM of H_2O_2 for additional 12 h (Ismail et al., 2012). The cell survival when 250 μM H_2O_2 was used alone was $\sim 40\%$, suggesting that 250 μM of H_2O_2 was able to induce approximately 60% cell death, and therefore it was applied for our subsequent experiments. However, the viability of the cells pretreated with different dosage of NS or NG were increased in dose dependent manner, indicating NS and NG protect PC-12 cells against H_2O_2 -induced cytotoxicity. NG showed a slightly higher protective activity as compared with NS (Figure 3B).

Effects of NS and NG on the activity of antioxidant enzymes

To investigate the antioxidant activity of NS and NG, the cellular antioxidant enzymes, CAT and SOD, were measured in the PC-12 cells that were pretreated with NS or NG for 12 h and subsequently exposed to 250 μM of H_2O_2 for another 12 h (Figure 4). When the PC-12 cells were treated with H_2O_2 alone, the activity of CAT and SOD were significantly decreased. However, treatments with TQ (positive control; 1 $\mu\text{g}/\text{mL}$) and NS and NG, increased the activity of these enzymes. The dosage of TQ was selected to conduct all

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Nigella glandulifera	C T T C G A T A A A A A A C G T A C C A A A T G G C A A T T T T T G T G G T A A T T T G A A A A T C C C G G G A A A C C T T C A A T T T T T T A A C C C C A T G T G C C C C C A A T G C C C T A G G T T G A G G C C A C T T C G G C T G G G C C T C C C C A A C G
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Nigella glandulifera	G C G T C T T T C C C C C C C T T T T T G G T G G G G G A A A C G A A A A T G G C C C C C G A A C C C C T T C G A G G C G C G G C C G C T T A A A A T A T T G T C C C C A C G A C A G T G G C C G C G T C A A G A G G T G G T G T T T T T G T C T T C C C C A
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Figure 1: Identification of *Nigella* species by DNA sequencing. Comparison of DNA sequences of internal transcribed spacer (ITS) region of *Nigella sativa* and *Nigella glandulifera* Freyn & Sint.

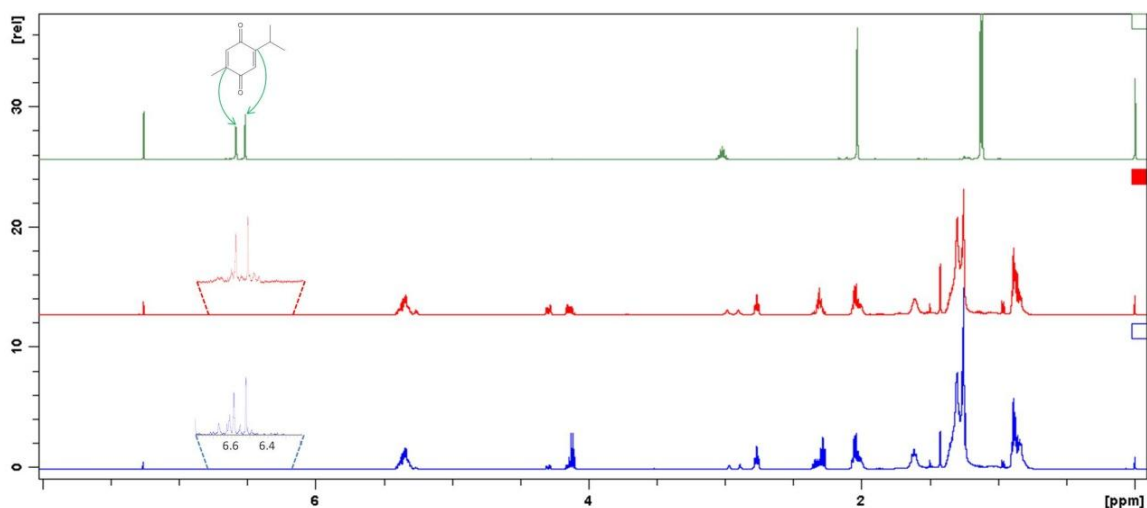


Figure 2: Quantification of thymoquinone in black seed by NMR spectra. ^1H NMR spectra of black seed. A. B.

Table 1: Calculation of TQ content in crude extracted oil and seed of *Nigella sativa* and *Nigella Glandulifera* Freyn & Sint.^a

Species	Sample no.	Seed (mg)	Oil extracted (mg)	TQ in oil (%)	TQ in seed (%)
<i>Nigella sativa</i>	1	500	120	0.704	0.169
	2	500	140	0.549	0.154
<i>Nigella glandulifera</i> Freyn & Sint	1	500	140	0.594	0.166
	2	500	130	0.788	0.205

^a Calculated by ^1H NMR.

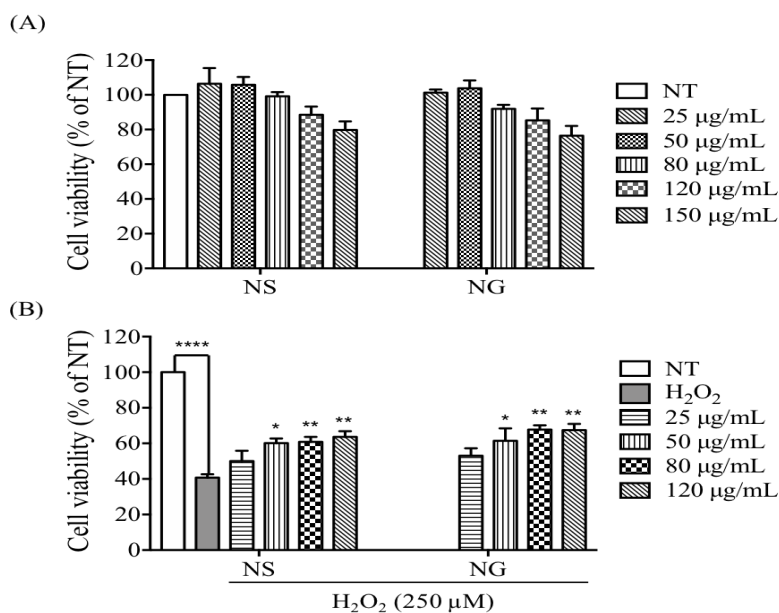


Figure 3: (A) Cell viability assay of PC-12 cells treated with NS and NG, respectively, for 24 h. Results are the mean \pm SEM. No significant difference between the control and treated groups. (B) Cell viability assay of PC-12 cells pretreated with NS or NG for 24 h and subsequent exposure to 250 μM H_2O_2 for 4 h. Results are the mean \pm SEM. No significant difference between the control and treated groups. Results are the mean \pm SEM. The symbol “*” indicates significant difference ($p < 0.05$) compared to H_2O_2 group.

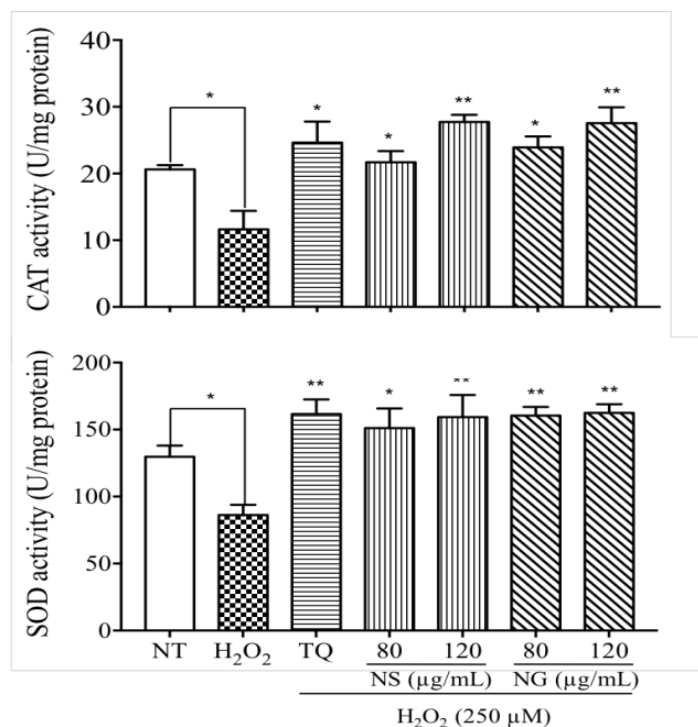


Figure 4: Effects of NS and NG on catalase (CAT) and superoxide dismutase (SOD) activity in hydrogen peroxide (H₂O₂)-treated PC-12 cells. Treatment with NS and NG showed a tendency to increase the activity of CAT and SOD in the H₂O₂-treated PC-12 cells. Results are the mean ± SEM. The symbol “*” indicates significant difference ($p < 0.05$) compared to H₂O₂ group.

experiments with the concentration of 85-90% cell survival rate by MTT assay (data not shown). The cellular activation of SOD treated with NG, which has higher content of TQ, was slightly higher than treatment with NS. But the activation of CAT was similar both in treatments with NS and NG. These results demonstrated that both NS and NG have ability to activate antioxidant enzymes on PC-12 cells and the antioxidant effect may be mediated to level of TQ of two herbs. No significant difference in the impact on antioxidant enzymes was observed between NS and NG.

Inhibition of ROS generation by NS and NG

We investigated whether NS and NG exert inhibitory effect on the production of ROS using DCF-DA in H₂O₂-treated PC-12 cells. The PC-12 cells pretreated with TQ, NS and NG, and subsequently incubated with H₂O₂ decreased H₂O₂-induced generation of intracellular ROS (Figure 5). NS showed weaker inhibition effect at a concentration of 80 μg/mL, but at concentration of 120 μg/mL, ROS production was significantly decreased. However, NG significantly attenuated ROS production in dose-dependent manner. Furthermore, inhibition effect of NG was much stronger than TQ and NS. These results suggest that NS and NG act as antioxidants which can directly scavenge excessive ROS generation in cells.

Preventive effect of NS and NG on H₂O₂-induced cell death in PC-12 cells

PC-12 cells were pretreated with 120 μg/mL of NS and NG, respectively, and further exposed to 250 μM H₂O₂ for 6 h. Then, calcein-AM/PI double staining assay was utilized for distinguishing viable cells from apoptotic and necrotic cells (Figure 6). After the cells were exposed with H₂O₂ alone, more amount of red fluorescence cells stained with PI (dead cells) were captured under microscope compared to NT group. However, the cells were pretreated with NS and NG, less amount of death cells was observed. Furthermore, treatment with NS or NG showed more prevention effect as compared with treatment with TQ alone. These results indicated that NS and NG have strong prevention effect against H₂O₂-induced cell death in PC-12 cells.

Effects of NS and NG on transcriptomic regulation of apoptotic and antioxidant genes in PC-12 cells exposed to H₂O₂

H₂O₂-treatment induced upregulation of antioxidant genes in PC-12 cells in contrast with the untreated cells (Figure 7). Pretreatment of the cells with NS or NG at 100 or 150 μg/mL prior to 250 μM H₂O₂ exposure upregulated Bcl-2,

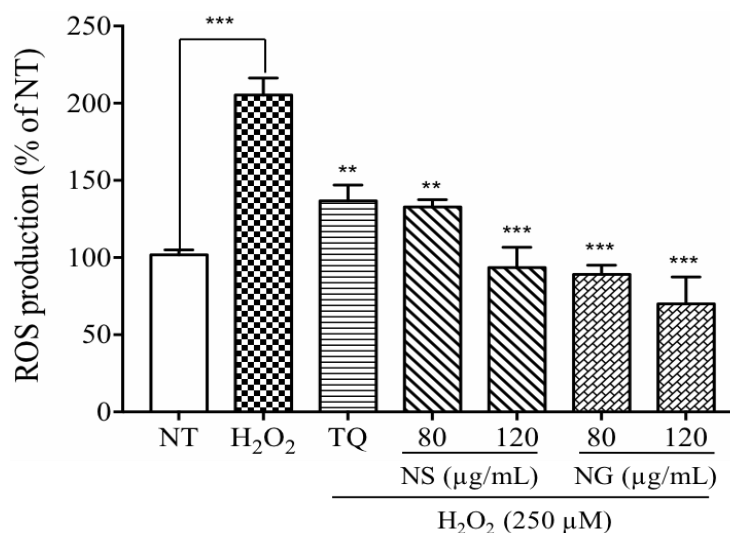


Figure 5: Effect of NS and NG on hydrogen peroxide (H₂O₂)-induced reactive oxygen species (ROS) production in PC-12 cells. Results are the mean ± SEM. The symbol “*” indicates significant difference ($p < 0.05$) compared to H₂O₂ group.

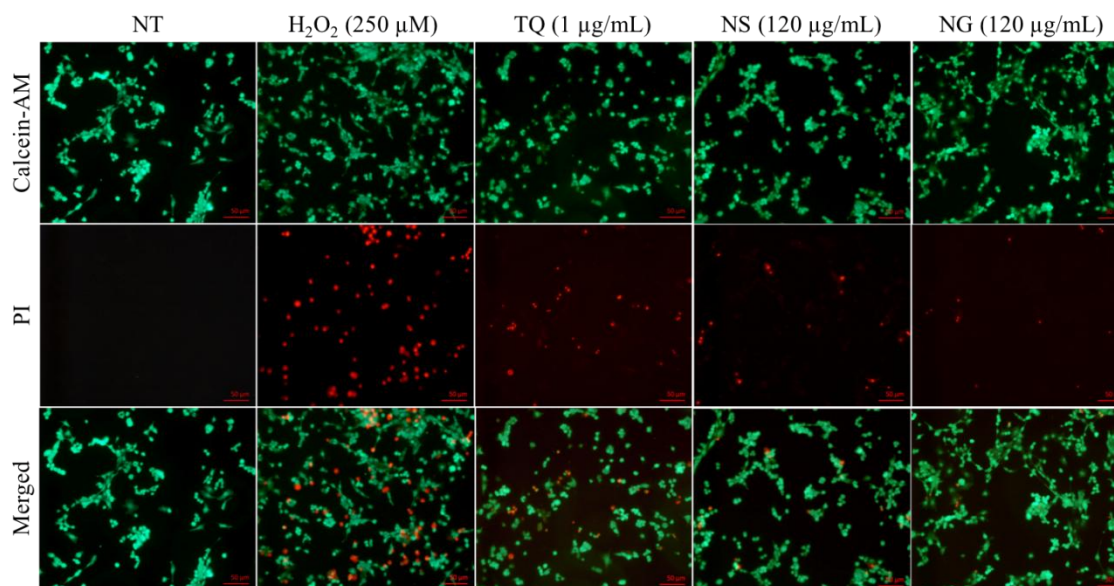


Figure 6: NS and NG prevented H₂O₂-induced cell death in PC-12 cells. PC-12 cells pretreated with TQ, NS and NG for 24 h followed by subsequent exposure to 250 µM H₂O₂ for 12 h. Viable cells are stained in green by calcein AM; Late apoptotic and necrotic cells are stained in red by propidium iodide (PI).

PARP, Akt and p38 MARK, and downregulated Bax, p53 and caspase 9 in dose dependent manner. However, a significant difference was observed for NF-κB and Jnk expression after exposure to 250 µM H₂O₂ as compared with non-treated cells, but no significant difference of those gene expression was observed in pretreatment of the extracted oils as compared with those treated with H₂O₂ alone. Additionally, pretreatment with 1 µg/mL TQ showed a difference for those two gene expressions as compared with H₂O₂ group.

The neuroprotective effect of the volatile oils of NS and NG may protect against neuronal cell death through activation of Bcl-2. Bcl-2 plays a crucial role in intracellular apoptotic signal transduction by regulating permeability of the mitochondria membrane. The reports suggested that the overexpression of Bcl-2 leads to reduced oxidative stress while decreased Bcl-2 expression leads to increased oxidative stress (Portt et al., 2011).

Mitochondria have been indicated as an early target of

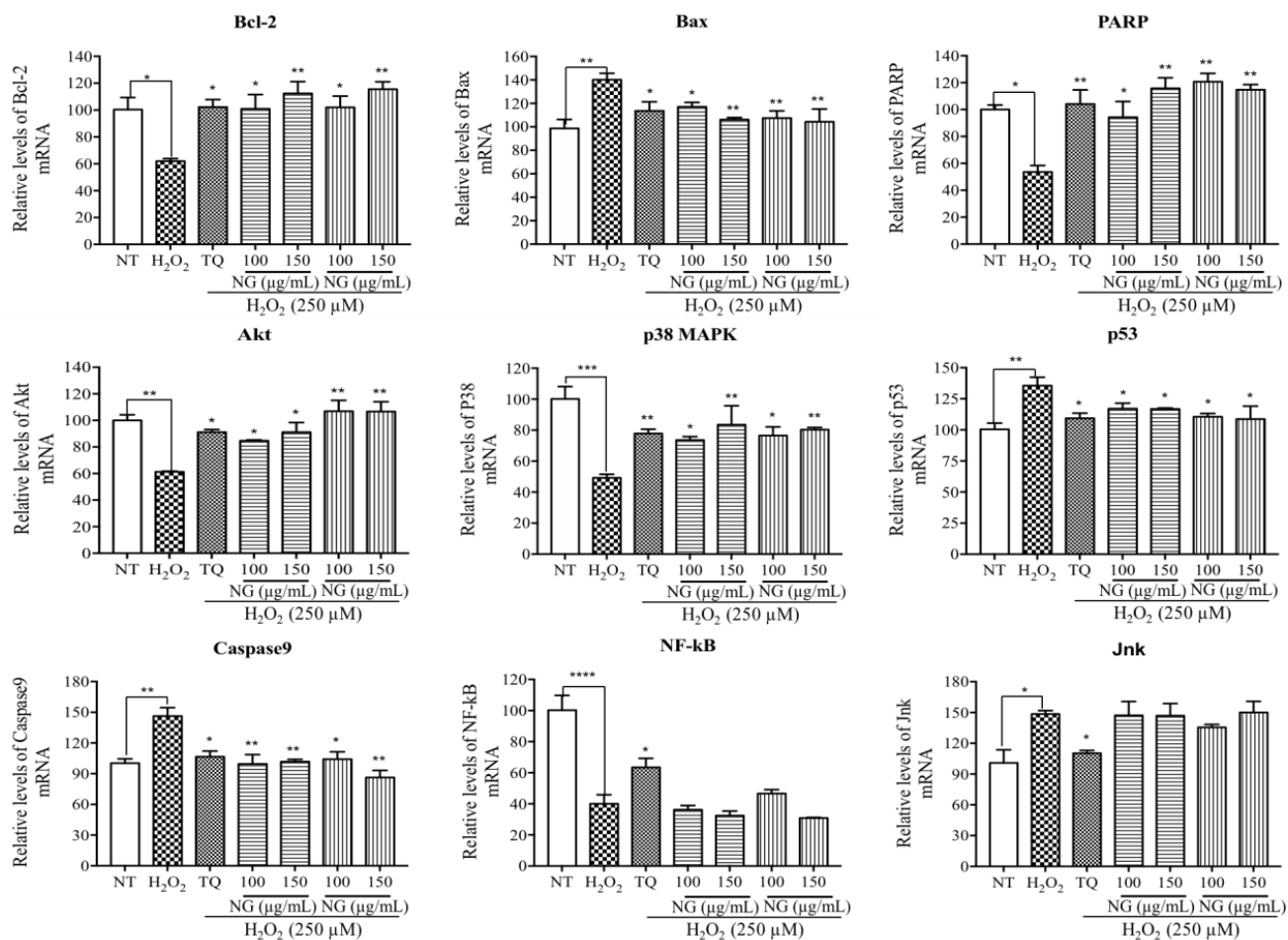


Figure 7: Expression of apoptotic genes, following treatment with NS (100 or 150 µg/mL) and NG (100 or 150 µg/mL) and subsequent exposure with 250 µM H₂O₂. Results are the mean ± SEM and “*” indicates significant difference at $p < 0.05$ versus H₂O₂.

oxidative damage, in which cause damage leads to cytochrome c that results in the formation of the apoptosome (Portt et al., 2011). The apoptosome activates initiator caspase, typically caspase 9, which leads to the activation of the executioner caspase 3. In response to apoptotic stimuli, pro-apoptotic members of Bcl-2 protein family (Bax and Bid) become activated and act on mitochondria to induce the release of cytochrome c. The other pro-apoptotic proteins are also released from the mitochondria including endonuclease G, the serine/protease Omi/HtrA2 and apoptotic inducing factor (AIF) (Wang and Youle, 2009). Cytochrome c in conjugation with Apaf-1, pro-caspase 9 and dATP forms the apoptosome that activates a caspase cascade and cleaves DNA repair enzymes including poly (ADP-ribose) polymerase (PARP), eventually leading to cellular damage and apoptosis (Portt et al., 2011; Sugawara et al., 2004). Notably, the major signaling pathway in response to oxidative stress include Akt pathway, mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-kB) signaling. Akt phosphorylates Bad and obviates its inhibitory effects on

Bcl-xL, ultimately inhibiting the release of cytochrome c by blocking the channel formation on the mitochondrial membrane by Bax. Akt also inhibits proteolytic activity of caspase-9 by phosphorylating it on Ser-196 (Sugawara et al., 2004). MAPK family members encompass a large number of serine/threonine kinases that play a critical role in the regulation of cell growth, differentiation, and cellular response to cytokines and stress. These include p38 kinases, extracellular signal-regulated kinase (ERK) and c-N-terminal kinases (Jnk) (Sugawara et al., 2004; Raingeaud et al., 1995). NF-kB regulates immune responses, inflammation, control of cell division and apoptosis, and its manipulation is reported to be valuable in treating ischemic stroke physical trauma to the brain or spinal cord, and neurodegenerative disorders including Alzheimer’s disease and Parkinson’s disease.

Conclusions

In this study, the volatile oils of NS and NG protected PC-12

cells against H₂O₂-mediated neurotoxicity by reducing cytotoxicity, inhibiting cellular apoptosis, and upregulating antioxidant and anti-apoptotic gene expressions and downregulating pro-apoptotic gene expressions. The result suggested that the volatile oils of NS and NG could protect PC-12 cells against oxidative stress-induced apoptosis.

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