

## NEUROPROTECTIVE ROLES OF ASIATICOSIDE ON HYDROGEN PEROXIDE-INDUCED TOXICITY IN SH-SY5Y CELLS

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### ABSTRACT

This study aims to determine whether the neuroprotective role of asiaticoside at maximum non-toxic dose (MNTD) or half MNTD ( $\frac{1}{2}$ MNTD) on  $H_2O_2$ -induced neurotoxicity in SH-SY5Y cells was mediated by regulation of reactive oxygen species (ROS) and nitric oxide (NO) through inducible nitric oxide synthase (iNOS) and heme-oxygenase 1 (HO-1). The levels of ROS, NO, iNOS and HO-1 in SH-SY5Y cells pre-treated with asiaticoside  $\frac{1}{2}$ MNTD were significantly reduced by 15.3, 55.6, 24.8 and 6.7 %, respectively as compared to the  $H_2O_2$  treatment group. Cells pre-treated MNTD only reduced the iNOS level significantly. Asiaticoside, particularly at  $\frac{1}{2}$ MNTD reduced the ROS, NO and iNOS levels. Contrarily, the HO-1 expression was down-regulated when treated with asiaticoside, which further suggest

**Keywords:** Asiaticoside; Heme-oxygenase 1; Inducible nitric oxide synthase; Nitric oxide; Reactive oxygen species.

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## 1. INTRODUCTION

Neurodegenerative diseases (ND) can be defined as hereditary and sporadic conditions which are characterised by progressive nervous system dysfunction. ND such as Alzheimer's disease, Parkinson's disease and multiple sclerosis are regularly associated with atrophy of the affected central or peripheral structures of the nervous system [1]. Today, ND remains as a worldwide issue which affects the ageing population. Statistics obtained from World Health Organization states that there are hundreds of millions people affected by neurological disorder globally. It is predicted that there are 35.6 million people in this world suffer from dementia and 7.7 million newly reported cases annually [1]. With this statistical report, many researchers are putting their efforts to reveal the aetiologies, understand the pathogenesis and discover novel therapeutic agents for ND. Among the patho-mechanisms, oxidative stress is considered to be the most prominent cause of ND [2].

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) against the antioxidant system of the body. ROS and RNS are the main species that cause oxidative stress in nervous system. The sources of ROS such as superoxide anion is mainly from the respiratory chain of mitochondria in human tissues whereas, nitric oxide (NO), a type of RNS, is gaseous free radical that acts as biological messenger as well as plays an essential role in the physiology of the central nervous system [3]. Central nervous system is reported to be particularly vulnerable to oxidative stress [4] due to a number of factors such as high utilisation of inhaled oxygen, susceptibility of oxidisable polyunsaturated fatty acid which is prone to lipid peroxidation and relative shortage of antioxidant defence systems [4]. Under inflammation and oxidative stress, inducible nitric oxide synthase (iNOS) is expressed and produces NO excessively to initiate the neurodegenerative process of neurons [5, 6]. When there is excessive production of NO, the heme-oxygenase 1 (HO-1), an antioxidant enzyme encoded in Antioxidant Response Element (ARE) will be expressed and modulates the antioxidative and anti-inflammatory effect through nuclear factor erythroid 2-related factor 2 (Nrf2)/ARE signalling pathway [7]. The understanding on effects of oxidative stress has led to the search of antioxidant compound that can attenuate and prevent oxidative damage on neuronal cells. One of such potential compound is asiaticoside. Asiaticoside is a triterpene glycoside present in *Centella*

*asiatica*, a plant used in the Ayurvedic medicine to treat a variety of illness such as diabetes, cough, dermatitis as well as for improving memory [8]. Previous studies on asiaticoside have shown many promising properties such as neuroprotection [9], anti-cancer [10] and anti-inflammatory [11]. Hence, asiaticoside is selected for this study as its neuroprotective effect and mechanisms of action on hydrogen peroxide ( $H_2O_2$ ) induced neurotoxicity is yet to be elucidated. Therefore, the main objective of this study was to determine the neuroprotective role of asiaticoside on  $H_2O_2$ -induced neurotoxicity in SH-SY5Y cells

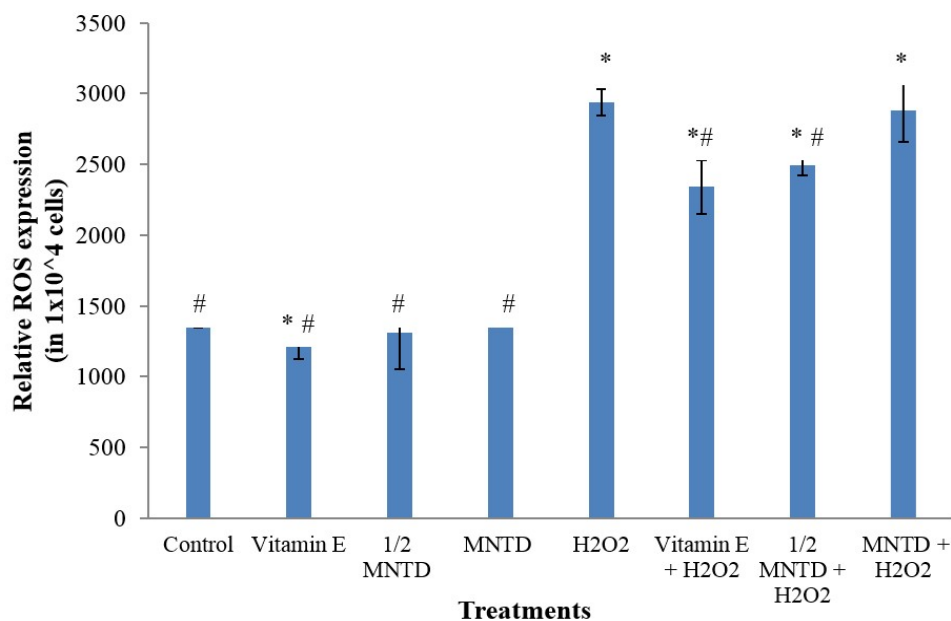
## 2. RESULTS AND DISCUSSION

### 2.1 Detection of Intracellular ROS Accumulation

The intracellular ROS has been reported to be involved in ND. Hence, the effects of asiaticoside at the MNTD and  $\frac{1}{2}$  MNTD on intracellular ROS levels in SH-SY5Y cells were evaluated in this study. The results revealed that the intracellular ROS levels were significantly increased regardless of the treatment groups compared to untreated cells when exposed to 100  $\mu M$  of  $H_2O_2$  (Fig. 1). This significant increase could be attributed to the characteristics of  $H_2O_2$ , which is highly diffusible, highly soluble and able to cross the plasma membrane. Once it passed through the plasma membrane, the cell's antioxidant defence systems counteracted with  $H_2O_2$  and subsequently produced ROS [2]. The study also showed a basal level of intracellular ROS was recorded in the untreated cells. This observation might be explained by under the normal physiological cellular respiration process, the respiratory chain of mitochondria in human tissue is responsible for the conversion of 1-2 % of the oxygen consumed to ROS [3].

The present study revealed that the cells pre-treated with  $\frac{1}{2}$  MNTD showed a significant reduction in intracellular ROS levels as compared to cell pre-treated with  $H_2O_2$  alone ( $p < 0.05$ ). This might be due to the antioxidative properties of asiaticoside as it was found capable of inhibiting or reducing  $H_2O_2$ -induced cell death and lowering intracellular free radical concentrations [12]. Nonetheless, the cells pre-treated with MNTD recorded a RFU reading at  $2882 \pm 226$ , which was not significantly differ with the intracellular ROS levels of the cells pre-treated with  $H_2O_2$  alone ( $2940 \pm 95$  RFU). In this case, MNTD may be a sub-cytotoxicity concentration to induce apoptosis. In addition, conversion of asiaticoside to asiatic acid via

hydrolytic cleavage might have occurred at MNTD as water, by product of the cellular respiration was present in the culture medium [13]. The conversion of asiaticoside to asiatic acid might increase the ROS levels in accordance with the studies by Park et al., who revealed that asiatic acid markedly increased the intracellular ROS level in SK-MEL-2 human melanoma cells [10].



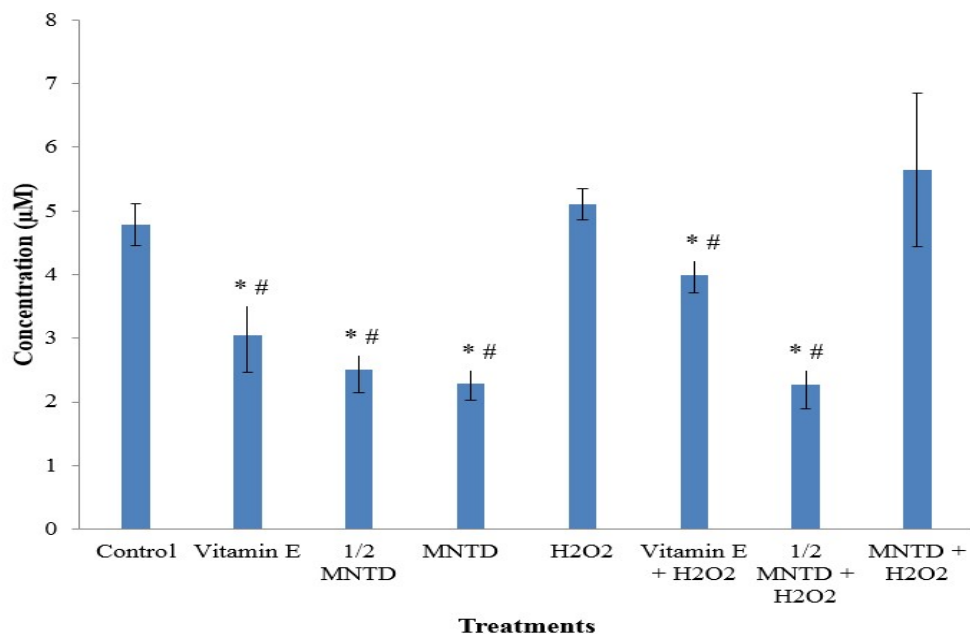
**Fig.1.** Effect of asiaticoside on intracellular reactive oxygen species (ROS) levels in SH-SY5Y cells. Data shown are the means  $\pm$  SD of triplicate. ‘\*’ denotes the treatment is significantly different from the untreated cells; ‘#’ denotes the treatment is significantly different from the H<sub>2</sub>O<sub>2</sub>- treated cells using Student’s t-test at  $p < 0.05$ .

## 2.2 Estimation of NO Level

As NO, a type of RNS is involved in ND, this study also estimated the effect of asiaticoside at the MNTD and  $\frac{1}{2}$  MNTD on NO level. As shown in Fig. 2, the NO level in SH-SY5Y cells was not significantly increased upon exposure to 100  $\mu$ M of H<sub>2</sub>O<sub>2</sub> regardless of the treatment groups compared to the untreated cells. This observation may be explained by the cells own antioxidant defence mechanism that might have counteracted with the NO produced. Hence, the production of NO was not increased significantly. The cells pre-treated with  $\frac{1}{2}$  MNTD

demonstrated a significant reduction in NO level from  $5.11 \pm 0.24$  to  $2.27 \pm 0.38$   $\mu\text{M}$  as compared to the cells treated with  $\text{H}_2\text{O}_2$  alone. Lee *et al.* stated that asiatic acid, the hydrolyses form of asiaticoside exerted significant neuroprotective effects on cultured cortical cells by potentiation of the cellular oxidative defence mechanism which involved in reducing NO level [14]. Apart from that, Guo *et al.* discovered the anti-inflammatory property of asiaticoside that inhibited NO synthesis and facilitated ulcer healing in rats with gastric ulcer [11].

On the other hand, the cells pre-treated with MNTD showed an increase in NO level ( $5.65 \pm 1.21$   $\mu\text{M}$ ) upon treatment with  $\text{H}_2\text{O}_2$ . Nevertheless, the data obtained did not show any significant different (at  $p < 0.05$ ) as compared to the control ( $4.78 \pm 0.33$   $\mu\text{M}$ ) and the cells treated with  $\text{H}_2\text{O}_2$  alone ( $5.11 \pm 0.24$   $\mu\text{M}$ ). In this case, the MNTD may serve as a sub-concentration to induce NO production on SH-SY5Y cells. Bhaumik *et al.* stated that asiaticoside induced NO production in peritoneal macrophages of *Leishmania donovani* in a concentration and time dependent manner and the maximal NO generation was observed with 25 mg/l asiaticoside at 36 h, suggesting that the increased NO in asiaticoside-treated group appeared to be related to the increase in NO synthase transcript in the cells [15].

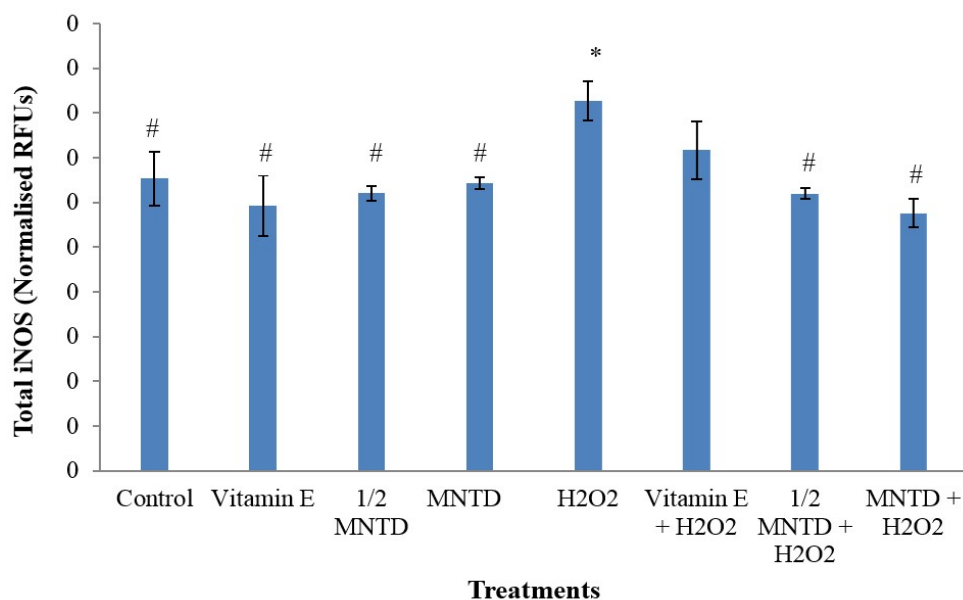


**Fig.2.** Effect of asiaticoside on nitric oxide (NO) levels in SH-SY5Y cells. Data shown are the means  $\pm$  SD of triplicate. ‘\*’ denotes the treatment is significantly different from the untreated cell; ‘#’ denotes the treatment is significantly different from the H<sub>2</sub>O<sub>2</sub>- treated cells using Student’s t-test at  $p < 0.05$ .

### 2.3 Measurement of iNOS Expression

As iNOS is reported to be involved in the production of NO by neurons [16, 17]. Thus, the effect of asiaticoside at the MNTD and 1/2MNTD on iNOS expression on SH-SY5Y cells was also measured. The results presented in Fig. 3 revealed that the cells treated with H<sub>2</sub>O<sub>2</sub> alone showed a significant increase in iNOS expression from  $0.131 \pm 0.012$  to  $0.165 \pm 0.009$  RFU as compared to the untreated group. Under oxidative stress, iNOS was expressed and produced NO excessively to initiate the neurodegenerative process of neuron [5, 6]. This may possibly explained why the iNOS expression level was increased when exposed to 100  $\mu$ M of H<sub>2</sub>O<sub>2</sub>. Nevertheless, the high level of iNOS can be counteracted by treating the SH-SY5Y cells with asiaticoside at 1/2 MNTD and MNTD, which brought a significant decrease from  $0.165 \pm 0.009$  to  $0.124 \pm 0.002$  and  $0.115 \pm 0.006$  RFU, respectively. The inhibition of iNOS activity by asiaticoside is in accordance with the studies by Guo *et al.* who stated the anti-inflammatory property of asiaticoside was through inhibition of iNOS activity [11].

Zhai *et al.* stated that oxidative stress in response to hyperglycemia might promote NO-over production in rat mesangial cells due to increased iNOS protein expression [18]. In general, the neuron produces NO mainly by a calcium dependent activation of neuronal NOS (nNOS) [16]. Nevertheless, some neurons also appear to express iNOS to produce NO [16, 17] as the nitric oxide synthase (NOS) are reported to have at least three isoform, nNOS identified in neurons, iNOS identified in glial cells and endothelial NOS [5, 19]. Hence, this may explain why the expression level of iNOS did not correlate with the NO level in the cells pre-treated with asiaticoside at MNTD as iNOS might not be the only NOS that contributed to the production of NO in SH-SY5Y cells.



**Fig.3.** Effect of asiaticoside on inducible nitric oxide synthase (iNOS) expression levels in SH-SY5Y cells. Data shown are the means  $\pm$  SD of triplicate. ‘\*’ denotes the treatment is significantly different from the untreated cell; ‘#’ denotes the treatment is significantly different from the H<sub>2</sub>O<sub>2</sub>-treated cells using Student’s t-test at  $p < 0.05$ .

#### 2.4 Measurement of HO-1 Expression

In response to oxidative stress, the HO-1 antioxidative protein will be expressed via the Nrf2/ARE signalling pathway to regulate the mitochondrial protection, cellular antioxidant and anti-inflammatory defence. Hence, the effect of asiaticoside at MNTD and 1/2 MNTD on

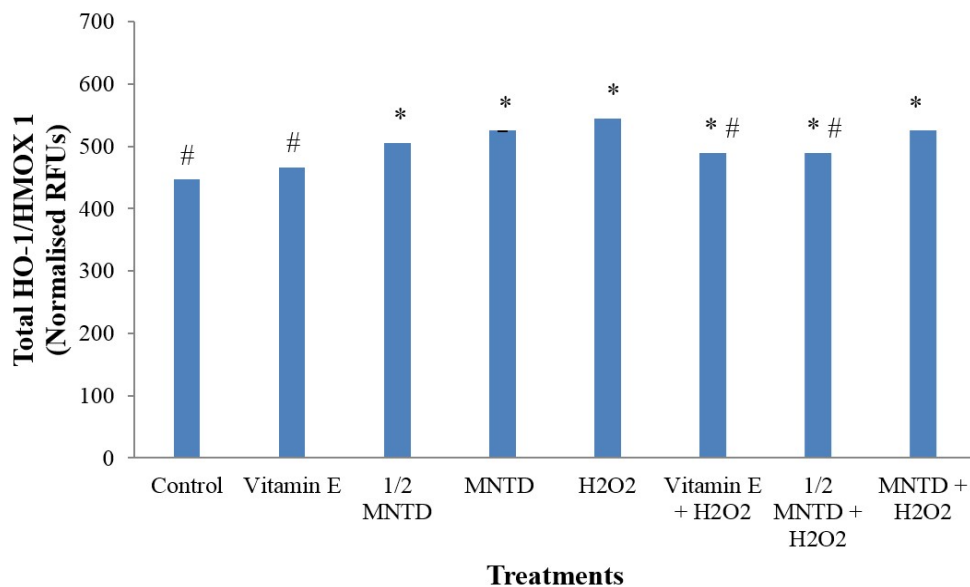
HO-1 expression in SH-SY5Y cells was measured and presented in Fig. 4. It was clearly observed that the cells treated with H<sub>2</sub>O<sub>2</sub> alone showed a significant increase in HO-1 expression compared to the untreated group from  $486.8 \pm 16.6$  to  $572.3 \pm 18.9$  RFU. Under the oxidative stress, the Nrf2, a transcription factor was translocated from the cytoplasm into the nucleus [20]. Nrf2 was then bind to the ARE and thus regulated the gene expression involved in the mitochondrial protection and cellular antioxidant and anti-inflammatory defence such as HO-1 [21]. Therefore, it brought about a significant increase in HO-1 expression in cells exposed to 100  $\mu$ M of H<sub>2</sub>O<sub>2</sub>.

Yang *et al.* stated that SH-SY5Y cells treated with CDDO methyl amide, a synthetic triterpenoid showed neuroprotective effect through the activation of Nrf2/ARE pathway and up-regulation of HO-1, NADPH quinine oxidoreductase-1 (NQO-1) and other ARE genes [22]. However, the present studies reported a contradict findings whereby the HO-1 expression was reduced when the SH-SY5Y cells were pre-treated with asiaticoside at MNTD ( $551.6 \pm 16.9$  RFU) and  $\frac{1}{2}$  MNTD ( $533.9 \pm 3.4$  RFU), of which the expression of the later was reduced significantly as compared to the H<sub>2</sub>O<sub>2</sub> treatment alone ( $572.3 \pm 18.9$  RFU). This observation might be due to the presence of Bach-1, which is a repressor of the oxidative stress response [23]. Okada *et al.* stated that Bach-1 depletion by small interfering RNAs or by deletion of Bach-1 enhanced HO-1 expression in the absence of H<sub>2</sub>O<sub>2</sub>, which indicating that Bach-1 is a critical repressor of HO-1 in keratinocytes [23].

The transcription factor Bach-1 is a repressor of the oxidative stress response in higher eukaryotes [23]. In the presence of oxidative stress, Bach-1 is inactivated [23] and the Nrf2 is released from Keap1, allowing Nrf2 to shuttle to the nucleus to initiate transcription by binding to the ARE as a heterodimer with a member of the small Maf family [24]. In addition, Bach-1 is able to form heterodimer with small Maf to bind to ARE as well, of which suggested that Bach-1 and Nrf2 would compete with each other to regulate the ARE-mediated gene expression [25]. Dhakshinamoorthy *et al.* also stated that when the human liver carcinoma cells, Hep-G2 were treated with tert-butylhydroquinone, an antioxidant, the accumulation of Bach-1 in the nucleus was significantly delayed over the Nrf2 [25]. The study also suggested that antioxidant-induced delayed accumulation of Bach-1 contributed to the down regulation of ARE-regulated gene, most likely by reducing the NQO-1 to normal



levels (Dhakshinamoorthy et al., 2005). Hence, it may explain why there was reduction of HO-1 expression when the cells were pre-treated with asiaticoside.



**Fig.4.** Effect of asiaticoside on heme-oxygenase 1 (HO-1) expression levels in SH-SY5Y cells.

Data shown are the means  $\pm$  SD of triplicate. ‘\*’ denotes the treatment is significantly different from the untreated cells; ‘#’ denotes the treatment is significantly different from the H<sub>2</sub>O<sub>2</sub>- treated cells using Student’s t-test at  $p < 0.05$ .

### 3. EXPERIMENTAL

#### 3.1 Preparation of Asiaticoside

The pure asiaticoside (Fluka, Germany) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to prepare a stock solution with the concentration of 100  $\mu$ g/mL. The stock was further diluted with Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) to the desired concentrations.

#### 3.2 Cell Culture

SH-SY5Y cell line (provided kindly by Dr Say Yee How, Universiti Tunku Abdul Rahman, Malaysia) was cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco, South America), 100 units/mL of penicillin and 100  $\mu$ g/mL streptomycin (Gibco, USA) and 0.1 % amphotericin B (Gibco, USA) in a 5 % carbon dioxide incubator at 37 °C.

### 3.3 Initiation of Treatments

To examine the effects of asiaticoside on SH-SY5Y cells, the cells at the density of  $4 \times 10^4$  cells per ml were seeded in 12-well transparent plate (Corning, USA) and incubated until 70 % confluent. The cells were assigned to a total of eight treatment groups (Table 1). Cells treated with 50  $\mu$ M of Vitamin E, D- $\alpha$ -tocopherol succinate (Sigma-aldrich, Japan) acted as the positive control. After 24 h of pre-treatment with maximum non-toxic dose (MNTD) or half MNTD ( $\frac{1}{2}$ MNTD), which were 0.54 and 0.27  $\mu$ g/mL, respectively, cells were exposed to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for another 24 h. After which, the levels of ROS, NO, iNOS and HO-1 were determined.

**Table 1.** Treatment groups used to investigate the neuroprotective role of asiaticoside on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced neurotoxicity in SH-SY5Y cells

Group	Treatment
1	Control (untreated cell)
2	H <sub>2</sub> O <sub>2</sub> only (100 $\mu$ M)
3	Asiaticoside MNTD (0.54 $\mu$ g/mL)
4	Asiaticoside $\frac{1}{2}$ MNTD (0.27 $\mu$ g/mL)
5	Vitamin E (D- $\alpha$ -tocopherol succinate) (50 $\mu$ M)
6	Asiaticoside MNTD (0.54 $\mu$ g/mL) + 100 $\mu$ M H <sub>2</sub> O <sub>2</sub>
7	Asiaticoside $\frac{1}{2}$ MNTD (0.27 $\mu$ g/mL) + 100 $\mu$ M H <sub>2</sub> O <sub>2</sub>
8	Vitamin E (D- $\alpha$ -tocopherol succinate) (50 $\mu$ M) + 100 $\mu$ M H <sub>2</sub> O <sub>2</sub>

### 3.4 Detection of Intracellular ROS Accumulation

After the treatment as mentioned, the DMEM medium was removed from the 12-well plate. Then, the treated and untreated cells were washed with phosphate buffered saline. Then, 100  $\mu$ L of 10 mM dichloro-dihydro-fluorescein diacetate (Sigma-aldrich, Israel) was added and incubated for 30 min at 37°C. After that, the cells were examined using fluorescence microplate reader (SpectraMax, USA) at excitation wavelength of 485 nm and emission wavelength of 538 nm. The intracellular ROS level of each treatment was expressed as relative fluorescence unit (RFU) per 10,000 cells.

### 3.5 Estimation of NO Levels

A total of 50  $\mu$ L of cell culture supernatant from all the treatment groups were assayed by equal volume of Griess reagent (Sigma-alrich, USA) and the mixture was incubated for 15 min [26]. The absorbance reading was measured at 540 nm using microplate reader (SpectraMax, USA), in which DMEM was used as the blank. The amount of nitrite in the sample was then estimated from a sodium nitrite standard curve constructed from 0 to 100  $\mu$ M.

### 3.6 Measurement of iNOS and HO-1 Expression

The expression level of iNOS and HO-1 were evaluated by using Enzyme-linked immunosorbent assay (ELISA) assay kits (R&D System Inc, USA) according to the manufacturer's protocols.

### 3.7 Statistical Analysis

All the experiments in this study were performed in triplicate and repeated once, unless otherwise stated. All data were expressed as mean  $\pm$  standard deviation (SD) and subjected to Student's t-test whereby  $p < 0.05$  was considered statistically significant.

## 4. CONCLUSION

In summary, the present study has demonstrated that asiaticoside attenuated the neurotoxicity by reducing the ROS, NO and iNOS, particularly when the cells were pre-treated with  $\frac{1}{2}$ MNTD. However, there was no up-regulation of HO-1 expression after the pre-treatment with asiaticoside. Thus, the exact neuroprotective mechanism of asiaticoside still requires further investigation. For the future investigations, the expression of Nrf2, Bach-1 and HO-1 mRNA shall be measured in a time dependant manner by reverse transcription polymerase chain reaction and western blot analysis as previous study has stated that antioxidant might induce the delay of Bach-1 accumulation in the nucleus. Hence, these suggested studies will probably give a clearer idea on how asiaticoside regulates the Nrf2/ARE signalling pathway.

## 5. ACKNOWLEDGEMENTS

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