



Neuroprotective effect of *Amaranthus lividus* and *Amaranthus tricolor* and their effects on gene expression of *RAGE* during oxidative stress in SH-SY5Y cells

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ABSTRACT. *Amaranthus* plants, or spinach, are used as food sources worldwide. *Amaranthus* leaves are rich in antioxidant compounds, which act as free radical scavengers. Oxidative stress caused by the aberrant production of reactive oxygen species (ROS) represents an important mechanism for neuronal dysfunction and cell loss in different neurodegenerative disorders. The neuroprotective effects of antioxidant-containing plants have been extensively demonstrated in different models of neurotoxicity. However, few studies have investigated the antioxidant properties of *Amaranthus* extracts and their effect on the nervous system. In the present study, the leaves of *Amaranthus lividus* and *Amaranthus tricolor* were extracted using petroleum ether, dichloromethane, and methanol. Results indicated that antioxidant activities were the highest in methanol extracts from

both kinds of *Amaranthus* leaves. In addition, oxidative stress was induced in human neuroblastoma cell lines (SH-SY5Y) by using H₂O₂. Intracellular oxidative stress, cytotoxicity, and gene expression of *RAGE* were then determined. *In vitro* results demonstrated that pretreatment with *A. lividus* and *A. tricolor* extracts can significantly decrease cell toxicity and intracellular ROS production in SH-SY5Y cells. Interestingly, the extracts also significantly downregulated the expression of oxidative stress genes such as *HMOX-1*, *RAGE*, and *RelA/NF-κB*. Our results suggested that *Amaranthus* leaves may be useful for reducing oxidative stress and may be beneficial for age-related diseases and neurodegenerative disorders.

Key words: *Amaranthus*; Oxidative stress; RAGE; Neuronal cells

INTRODUCTION

Amaranthus (Amaranthaceae) are resilient plants that grow under a wide range of climatic conditions. *Amaranthus* sp, more commonly known as spinach, is one of the most popular leafy vegetables consumed worldwide. Four types of *Amaranthus* vegetables are native to Thailand: *A. viridis*, *A. lividus*, *A. spinosa*, and *A. tricolor* (Council, 1983). We as consumers take advantage of the many uses of *Amaranthus* by producing flour from *Amaranthus* seeds, making salads from fresh leaves, using inflorescences as a source of natural red dye, and utilizing its waste products as animal foodstuff (López-Mejía et al., 2014). Amaranth leaves are rich sources of antioxidants, protein, carotenoids, vitamin C, dietary fiber, and minerals such as calcium, iron, zinc, and magnesium (Shukla et al., 2006; Ozsoy et al., 2009; Anitha and Ponbavani, 2013; López-Mejía et al., 2014). Amaranth oil is highly unsaturated, contains about 70% oleic acid, and is a rich source of squalene and tocotrienols (Tikekar et al., 2008).

Various environmental stresses induce excessive production of reactive oxygen species (ROS), which can lead to detrimental effects on cellular proteins, membrane lipids, and nucleic acids, ultimately resulting in cell death. On the other hand, ROS are also well-described second messengers in a variety of cellular processes. A fine balance exists between the production of ROS and ROS scavengers, which are upregulated during various environmental stresses via non-enzymatic and enzymatic antioxidants (Uttara et al., 2009). Oxidative stress plays a major role in the pathogenesis of many degenerative diseases such as cancer, cardiovascular disease, osteoporosis, aging, as well as neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Halliwell and Gutteridge, 1984; Sian et al., 1994; Multhaup et al., 1997; Halliwell, 2006; Wang and Michaelis, 2010). It was suggested that increased ROS production activates several pathways involved in disease complications such as increased formation of advanced glycation end-products (AGEs) and AGE receptors (RAGE) (Ramasamy et al., 2005). Both RAGE activation and oxidative damage have been implicated in chronic degenerative diseases including type 2 diabetes mellitus, cancer, and cardiovascular diseases (Jiang et al., 2004; Tan et al., 2007).

Traditional medicine uses a wide range of natural products to treat various disorders, and recently, increasing interest has been generated in this field due to the health benefits it provides. Products containing antioxidants have been used as part of several drug preparations for the treatment of diseases. Fruits and vegetables are the most important sources of

antioxidants, and interests in finding new natural antioxidants have been on the rise over the last few years. It is believed that these natural antioxidants can play an important role in the biological systems by scavenging free radicals and chelating catalytic metals (Halliwell and Gutteridge, 1984). Using neuronal immortalized SH-SY5Y cell lines, we have evaluated the neuroprotective effects of *Amaranthus* extracts, as well as their effect on gene expression of *RAGE* during H₂O₂-induced oxidative stress. Our data clearly demonstrated that *Amaranthus* extracts were able to reduce oxidative stress by suppressing the expression of *HMOX-1*, *RAGE*, and *RelA*.

MATERIAL AND METHODS

Material

Cell culture

The human neuroblastoma cell line SH-SY5Y, a kind gift from Dr. Tewarit Sarachana (Faculty of Allied Health Sciences, Chulalongkorn University), was used for all experiments. The SH-SY5Y cell line is a neuronal subclone derived from the SK-N-SH cell line. The cells were cultured in a humidified, 5% (v/v) CO₂-controlled environment at 37°C, and grown in MEM/F12 culture medium supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Confluent cultures were used for experiments, and were made quiescent when appropriate by a 24-h incubation with medium supplemented with 5% FBS.

Plant materials and extract preparation

Amaranthus lividus and *Amaranthus tricolor* were collected from a single source at Mahidol University, Salaya, Nakhon Pathom Province, Thailand. They were authenticated at the Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand, with Voucher No. 013695 (BCU) for *A. tricolor* and No. 013696 (BCU) for *A. lividus*. The plants were successively extracted using a Soxhlet extractor. Briefly, 30 g of the plant powder was extracted in a series of organic solvents (1:10, w/v) with increasing polarity (petroleum ether, dichloromethane, and methanol). The plant extracts were concentrated in a vacuum rotary evaporator under reduced pressure, and were dissolved in dimethyl sulfoxide (DMSO). They were stored as 100 mg/mL stock solutions at -20°C in the dark.

Reagents

Eagle's minimum essential medium (MEM) with Earle's balanced salts (MEM/EBSS), Ham's F12 nutrient mixture, FBS, Hank's balanced salt solution (HBSS), and antibiotic supplement were purchased from HyClone (Logan, UT, USA). 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (vitamin C), methanol, petroleum ether, dichloromethane, and DMSO were purchased from Merck (Darmstadt, Germany). CellTiter 96® Aqueous One solution cell proliferation assay (MTS) and CytoTox 96® non-radioactive cytotoxicity assay

(LDH) were purchased from Promega (Madison, WI, USA). 2',7'-DCFH-DA was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TRI reagent was purchased from Favorgen Biotech (Taiwan, China). The ImProm-II reverse transcription system and deoxyribonuclease I (DNase I) were purchased from Promega. The AccuPower® 2X Greenstar™ qPCR master mix and primers were all purchased from Bioneer Corporation (Daejeon, Republic of South Korea). All other reagents were of analytical grade.

Methods

Determination of antioxidant activities

The DPPH assay was performed via generation of the stable free radical DPPH (DPPH•). DPPH was dissolved in absolute methanol. Fresh DPPH solution was prepared daily, and its absorbance at 517 nm was adjusted to 0.650 ± 0.020 using absolute methanol. Thereafter, 20 μL *Amaranthus* extracts (1 mg/mL) or ascorbic acid (serving as a standard) was mixed with 180 μL DPPH solution, and incubated at room temperature for 30 min in the dark. The absorbance of the reaction mixture was measured at 517 nm, and the results are reported as mg vitamin C equivalent antioxidant capacity (VCEAC)/g dry plant material. The % of scavenging activity was calculated using the following formula:

$$\% \text{DPPH scavenged} = 100 \times [\text{Abs. control} - (\text{Abs. sample} - \text{Abs. blank sample}) / \text{Abs. control}]$$

Abs. control was the absorbance of DPPH solution, *Abs. sample* was the absorbance of the plant sample extracts as well as standard compound (ascorbic acid), and *Abs. blank sample* was the absorbance of the sample in absolute methanol.

In the ABTS assay, ABTS• + solution was produced. Briefly, 8 mL 7.4 mM ABTS• + and 12 mL 2.45 mM potassium persulfate solution was mixed and incubated for 16-18 h at room temperature in the dark. The ABTS• + solution was diluted with absolute methanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. The assay was carried out by mixing 20 μL *Amaranthus* extracts (1 mg/mL) or ascorbic acid (serving as a standard) with 180 μL ABTS• + solution for 45 min in the dark. Absorbance of the reaction mixture was measured spectrophotometrically at 734 nm. Results are reported as mg VCEAC/g dry plant material. The % scavenging activity was calculated using the following formula:

$$\% \text{ABTS scavenged} = 100 \times [\text{Abs. control} - (\text{Abs. sample} - \text{Abs. blank sample}) / \text{Abs. control}]$$

Cell viability

Viability of SH-SY5Y cells was assessed using the CellTiter 96® AQueous One solution cell proliferation assay. The assay is based on the bioreduction of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] into a soluble colored formazan product. This conversion is accomplished by either NADPH or NADH, which are produced by dehydrogenase enzymes in metabolically active cells. Briefly, following cell treatments, 20 μL MTS solution in 100 μL culture medium was added to SH-SY5Y cells and incubated at 37°C in 5% CO₂ for 1-4 h. Absorbance was measured at 490 nm.

Cytotoxicity

Cytotoxicity of SH-SY5Y cells was assessed using the CytoTox 96[®] non-radioactive cytotoxicity assay. The assay is based on the quantification of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. LDH released into the media is measured with a 30-min enzymatic assay, which converts a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Briefly, following cell treatments, 50 μ L supernatants in each well was pipetted onto a fresh microtiter plate with a set of control and lysed cells (to determine the background, and maximum release of LDH). Assay substrate solution (50 μ L) was added to each well, and the plate was then incubated in the dark for 30 min. Stop solution (50 μ L) was used to terminate the reaction, and absorbance was measured at 590 nm.

Measurement of intracellular ROS

Intracellular oxidative stress of SH-SY5Y cells was determined through the DCFH-DA assay. The assay employs a cell-permeable fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. In brief, following cell treatments, media were removed from all wells, and the cells were washed with warm HBSS. This was followed by the addition of 10 μ M DCFH-DA, and cells were incubated for 45 min at 37°C in 5% CO₂. The cells were then washed 3X with warm HBSS, and fluorescent intensity was measured using excitation and emission wavelengths of 485 and 535 nm, respectively. HBSS was used as a blank. The % cellular oxidative stress was calculated according to the following formula:

$$\text{Cellular oxidative stress (\%)} = \left[\frac{\text{fluorescence of treatment group} - \text{blank}}{\text{fluorescence of control group} - \text{blank}} \right] \times 100$$

Quantitative-reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from SH-SY5Y cells using the TRI reagent according to manufacturer instructions. RNA was treated with deoxyribonuclease I (DNase I; Promega) during isolation procedure. DNase I-treated RNA was reverse transcribed using the ImProm-II reverse transcription system according to manufacturer protocol. Quantification of mRNA was carried out using qPCR with SYBR green and the following specific primers: *RAGE* forward, 5'-GTGGGGACATGTGTGTCAGAGGGAA-3'; *RAGE* reverse, 5'-TGAGGAGAGGGCTGGCAGGGACT-3'; *HMOX-1* forward, 5'-GACCCATGACACCAAGGACC-3'; *HMOX-1* reverse, 5'-GGATGTGCTTTTCGTTGGGG-3'; *RelA* forward, 5'-TGGCCCCTATGTGGAGATCA-3'; *RelA* reverse, 5'-AGGGGTTGTTGTTGGTCTGG-3'; *GADPH* forward, 5'-TGCACCA CCAACTGCTTAGC-3'; *GADPH* reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. The cycling protocol was as follows: denaturation (95°C for 10 min), 40 cycles of amplification (95°C for 15 s, 60°C for 15 s), and a final extension at 72°C. A melting curve analysis was also performed to ensure the specificity of the amplified product. The expression for each gene was normalized to that of the control gene, *GAPDH*. Expression was quantified as fold-change using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$).

Statistical analysis

All experiments were repeated at least three times in triplicates or quadruplicates, as indicated. Data are reported as means \pm SE. Statistical significance between two groups was determined using the Student *t*-test; statistical significance between more than two groups was evaluated by one-way analysis of variance (ANOVA) using the SPSS version 20.0 software, followed by least significant difference statistical tests. A two-tailed P value was obtained, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Antioxidant activity of *A. lividus* and *A. tricolor* extracts

The radical scavenging abilities of *A. lividus* and *A. tricolor* extracts dissolved in petroleum ether, dichloromethane, and methanol were assessed via the ABTS and DPPH assays (Table 1). The methanol extract of *A. tricolor* appeared to have the most radical-scavenging activity in the ABTS radical scavenging assay at 25.03 ± 1.58 mg VCEAC/g extract. The dichloromethane and methanol extracts of *A. lividus* had the second highest radical-scavenging activity in the ABTS assay (20.90 ± 0.70 and 20.89 ± 0.69 mg VCEAC/g extract, respectively), and the *A. tricolor* petroleum ether extract had the least scavenging activity (2.07 ± 0.95 mg VCEAC/g extract). The DPPH radical scavenging assays showed that the methanol extract of *A. lividus* had the greatest radical-scavenging activity (15.18 ± 1.59 mg VCEAC/g extract), followed by the methanol extract of *A. tricolor* (13.86 ± 0.28 mg VCEAC/g extract), and the petroleum ether extract of *A. tricolor* had the least scavenging activity (1.73 ± 0.86 mg VCEAC/g extract).

Table 1. Antioxidant capacity of *Amaranthus lividus* and *Amaranthus tricolor* extracts.

Extracts	Antioxidant activity (mg VCEAC/g dry plant material)	
	ABTS assay	DPPH assay
<i>A. tricolor</i> (petroleum ether extract)	2.07 ± 0.95	1.73 ± 0.86
<i>A. tricolor</i> (dichloromethane extract)	17.90 ± 1.55	7.66 ± 0.68
<i>A. tricolor</i> (methanol extract)	25.03 ± 1.58	13.86 ± 0.28
<i>A. lividus</i> (petroleum ether extract)	3.87 ± 0.79	3.43 ± 1.13
<i>A. lividus</i> (dichloromethane extract)	20.90 ± 0.70	6.81 ± 1.93
<i>A. lividus</i> (methanol extract)	20.89 ± 0.69	15.18 ± 1.59

Data are reported as means \pm SE.

Effect of *A. lividus* and *A. tricolor* extracts on viability and oxidative stress in SH-SY5Y cells

The neuroprotective effect of *A. lividus* and *A. tricolor* extracts against H₂O₂-induced oxidative stress was investigated in neuroblastoma SH-SY5Y cells. The effect of *A. lividus* and *A. tricolor* extracts on SH-SY5Y cell viability was assessed by the MTS assay (Figure 1A and B). SH-SY5Y cells were incubated with various concentrations of *A. lividus* and *A. tricolor* extracts for 24 h. The result showed that petroleum ether, dichloromethane, and methanol extracts of *A. lividus* and *A. tricolor* at 1000 μ g/mL significantly reduced the numbers of living cells ($P < 0.05$). There was also a significant reduction ($P < 0.05$) in cell viability when they were treated with *A. lividus* extracts at a concentration of 500 μ g/mL in all solvents used for

extraction, and with methanol extracts at a concentration of 100 $\mu\text{g/mL}$. In addition, *A. tricolor* extracts at 500 $\mu\text{g/mL}$ in both dichloromethane and methanol caused significant reduction in cell viability ($P < 0.05$). In addition, the cell viability was $>80\%$ when cells were incubated with petroleum ether, dichloromethane, and methanol extracts of *A. lividus* and *A. tricolor* at $<100 \mu\text{g/mL}$, with the exception of the *A. lividus* dichloromethane extract, which showed $>80\%$ viability with a concentration of $<50 \mu\text{g/mL}$.

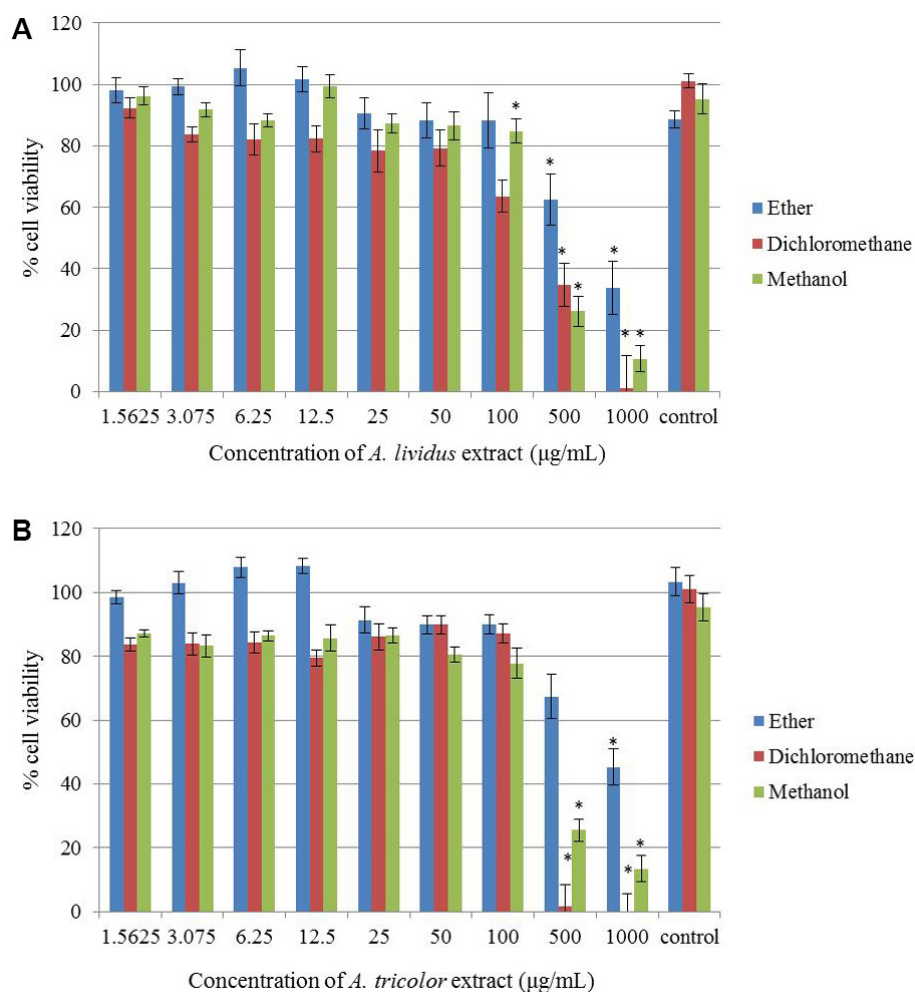


Figure 1. Effect of *Amaranthus lividus* and *Amaranthus tricolor* extracts on the viability of SH-SY5Y cells. SH-SY5Y cells were incubated with different concentrations of *A. lividus* and *A. tricolor* extracts (0-1000 $\mu\text{g/mL}$) for 24 h. The viability of SH-SY5Y cells treated with **A.** *A. lividus* and **B.** *A. tricolor* was assessed via the MTS assay. Values are reported as means \pm SE, which are depicted by vertical bars. All experiments were performed in triplicates ($N = 3$). * $P < 0.05$ is considered statistically significant as compared to the control (no treatment).

ROS levels are essential parameters of oxidative stress (Liu et al., 2010). To evaluate the protective effect of *A. lividus* and *A. tricolor* extracts on cells during oxidative stress, we

assessed ROS levels in SH-SY5Y cells using the intracellular fluorescent ROS dye, DCFH-DA, during H_2O_2 -induced oxidative stress. As shown in Figure 2, treatment of $10 \mu M H_2O_2$ significantly increased intracellular ROS levels ($P < 0.05$). Pretreatment with petroleum ether, dichloromethane, and methanol extracts of *A. tricolor* at $100 \mu g/mL$ significantly reduced ROS production ($P < 0.05$). Furthermore, both the petroleum ether and the methanol extract of *A. lividus* at $100 \mu g/mL$ significantly decreased intracellular ROS level, whereas the *A. lividus* dichloromethane extracts were effective in reducing intracellular ROS in SH-SY5Y cells at $50 \mu g/mL$ ($P < 0.05$). Therefore, our results indicated that *A. lividus* and *A. tricolor* extracts can effectively decrease the ROS accumulation in SH-SY5Y cells.

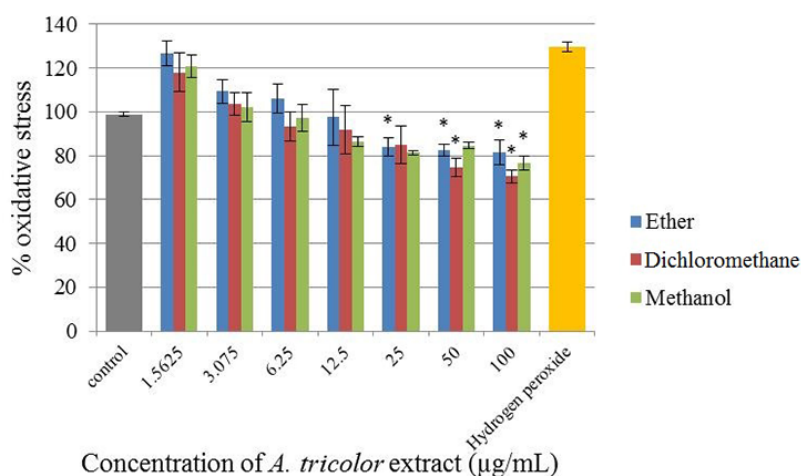
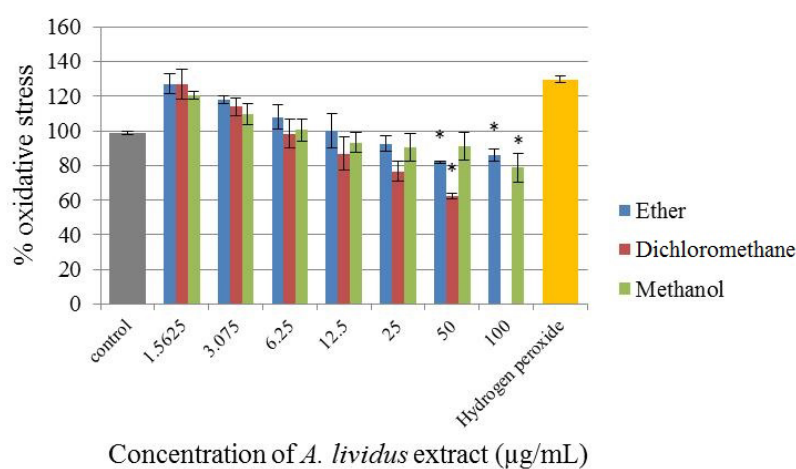


Figure 2. Reductive effect of *Amaranthus lividus* and *Amaranthus tricolor* extracts on oxidative stress in SH-SY5Y cells. SH-SY5Y cells were pre-incubated with different concentrations of **A.** *A. lividus* and **B.** *A. tricolor* extracts for 24 h prior to treatment with $10 \mu M H_2O_2$. Intracellular ROS levels were analyzed 24 h later using the DCFH-DA assay. Values are reported as means \pm SE, which are depicted by vertical bars. All experiments were performed in triplicate ($N = 3$). * $P < 0.05$ is considered statistically significant as compared to H_2O_2 -treated cells.

Protective effects of *A. lividus* and *A. tricolor* extracts on neurotoxicity in SH-SY5Y cells

To determine the extent of protection that *A. lividus* and *A. tricolor* extracts provide against H_2O_2 -induced oxidative damage in SH-SY5Y cells, LDH assays were performed. SH-SY5Y cells were incubated with different concentrations of *A. lividus* and *A. tricolor* extracts, and exposed to $10 \mu M H_2O_2$ for another 24 h. The LDH assay measures release of LDH as a result of cell membrane damage. As shown in Figure 3, overnight treatment with H_2O_2 resulted in a significant increase in LDH level as compared with controls ($P < 0.05$). Pretreatment with petroleum ether (50-100 $\mu g/mL$), dichloromethane (25-50 $\mu g/mL$) and methanol (25-100 $\mu g/mL$) *A. lividus* extracts resulted in a significant reduction in LDH release as compared with H_2O_2 alone ($P < 0.05$; Figure 3A). When pretreated with petroleum ether, dichloromethane, and methanol *A. tricolor* extracts at concentrations of 12.5-100 $\mu g/mL$, there was also a significant reduction in LDH release as compared with H_2O_2 treatment alone. These results showed that *A. lividus* and *A. tricolor* extracts can effectively prevent H_2O_2 -induced oxidative stress damage.

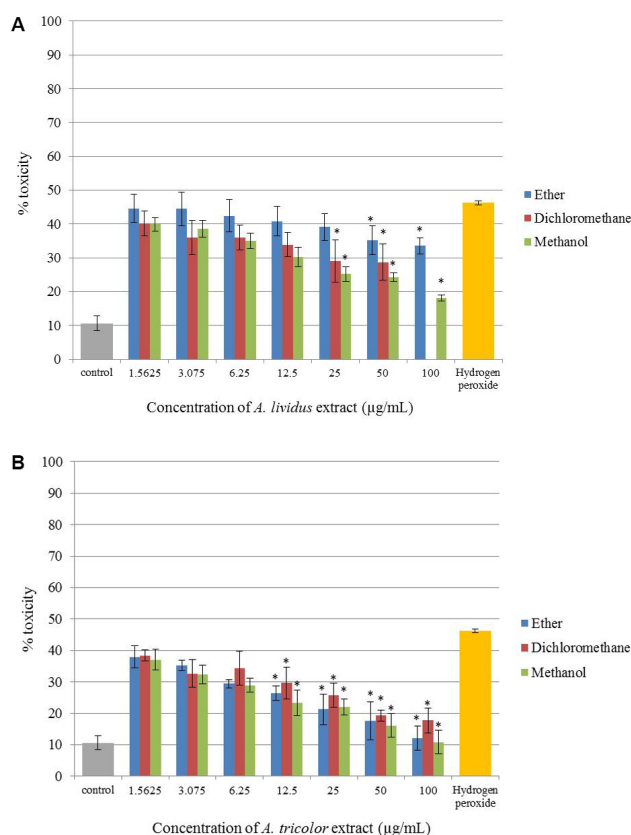


Figure 3. Protective effect of *A. lividus* and *Amaranthus tricolor* extracts on toxicity of SH-SY5Y cells. SH-SY5Y cells were pre-incubated with different concentrations of **A.** *A. lividus* and **B.** *A. tricolor* extracts for 24 h prior to treatment with $10 \mu M H_2O_2$. Cell toxicity was assessed 24 h later using the LDH assay. Values are reported as means \pm SE, as depicted by vertical bars. All experiments were performed in triplicate ($N = 3$). * $P < 0.05$ is considered statistically significant as compared to H_2O_2 -treated cells.

Effect of *A. lividus* and *A. tricolor* extracts on expression of *HMOX-1*, *RAGE*, and *RelA* in SH-SY5Y cells

To determine the protective effect of *A. lividus* and *A. tricolor* extracts at a molecule level, expression level of oxidative stress genes was determined by qPCR. Results showed that pretreatment with 25-100 µg/mL petroleum ether, 25-50 µg/mL dichloromethane, and 25-100 µg/mL methanol extracts of *A. lividus* resulted in significantly reduced *HMOX-1* expression in SH-SY5Y cells as compared with H₂O₂ treatment alone ($P < 0.05$; Figure 4A). Similarly, when pretreated with 50-100 µg/mL petroleum ether, and 25-100 µg/mL dichloromethane and methanol extracts of *A. tricolor*, expression of the *HMOX-1* was also significantly downregulated (Figure 4B). This confirmed that *A. lividus* and *A. tricolor* extracts could effectively prevent oxidative stress damage induced by H₂O₂.

As shown in Figure 4C, we found that pretreatment with 100 µg/mL petroleum ether, 50 µg/mL dichloromethane, and 50-100 µg/mL methanol extracts of *A. lividus* significantly reduced *RAGE* expression in H₂O₂-induced cells ($P < 0.05$). In addition, pretreatment with 100 µg/mL petroleum ether, 50-100 µg/mL dichloromethane, and 25-100 µg/mL methanol extracts of *A. tricolor* also led to significant downregulation of *RAGE* gene expression during H₂O₂ treatment ($P < 0.05$; Figure 4D). Therefore, *A. lividus* and *A. tricolor* extracts can effectively decrease *RAGE* gene expression in SH-SY5Y cells.

As upregulation of *RAGE* is followed by activation of the NF-κB pathway (Lander et al., 1997), mRNA expression of *RelA*, a protein involved in the NF-κB pathway, was measured. SH-SY5Y cells pre-incubated with *A. lividus* petroleum ether and methanol extracts at concentrations of 50-100 µg/mL and dichloromethane extract at concentrations of 25-50 µg/mL showed significantly reduced *RelA* expression during H₂O₂ treatment ($P < 0.05$; Figure 4E). Similarly, as demonstrated in Figure 4F, *RelA* expression in cells pre-incubated with *A. tricolor* extracts in petroleum ether and dichloromethane (100 µg/mL) as well as in methanol (50-100 µg/mL), was significantly decreased as compared with control cells ($P < 0.05$). This suggested that *A. lividus* and *A. tricolor* extracts can effectively decrease NF-κB activation in SH-SY5Y cells.

DISCUSSION

In this study, we assessed the antioxidant activities of *Amaranthus* via the ABTS radical-scavenging and DPPH radical-scavenging assays. Results from the ABTS radical-scavenging assay of *A. lividus* and *A. tricolor* extracts suggested that methanol extract of *A. tricolor* had the highest antioxidant activity, followed by dichloromethane and methanol extracts of *A. lividus*. On the other hand, *A. tricolor* petroleum ether extract had the least scavenging activity, as assessed by the ABTS radical-scavenging assay. In addition, *Amaranthus* extracts also demonstrated the ability to act as hydrogen-donors in the presence of DPPH stable radicals. As shown in Table 1, the scavenging activities of the extracts in the DPPH assays were similar to the results from the ABTS assays. The DPPH scavenging activities of the methanol extract of *A. lividus* exhibited the strongest antioxidant activity, followed by the methanol extract of *A. tricolor*. Again, the petroleum ether *A. tricolor* extract showed the weakest scavenging activity. The antioxidant capacity of various extracts was dependent on solvent polarity, which alluded to the polar nature of the active compound(s) in *Amaranthus*.

The antioxidant activity in *Amaranthus* sp was reported in previous studies (Ozsoy et

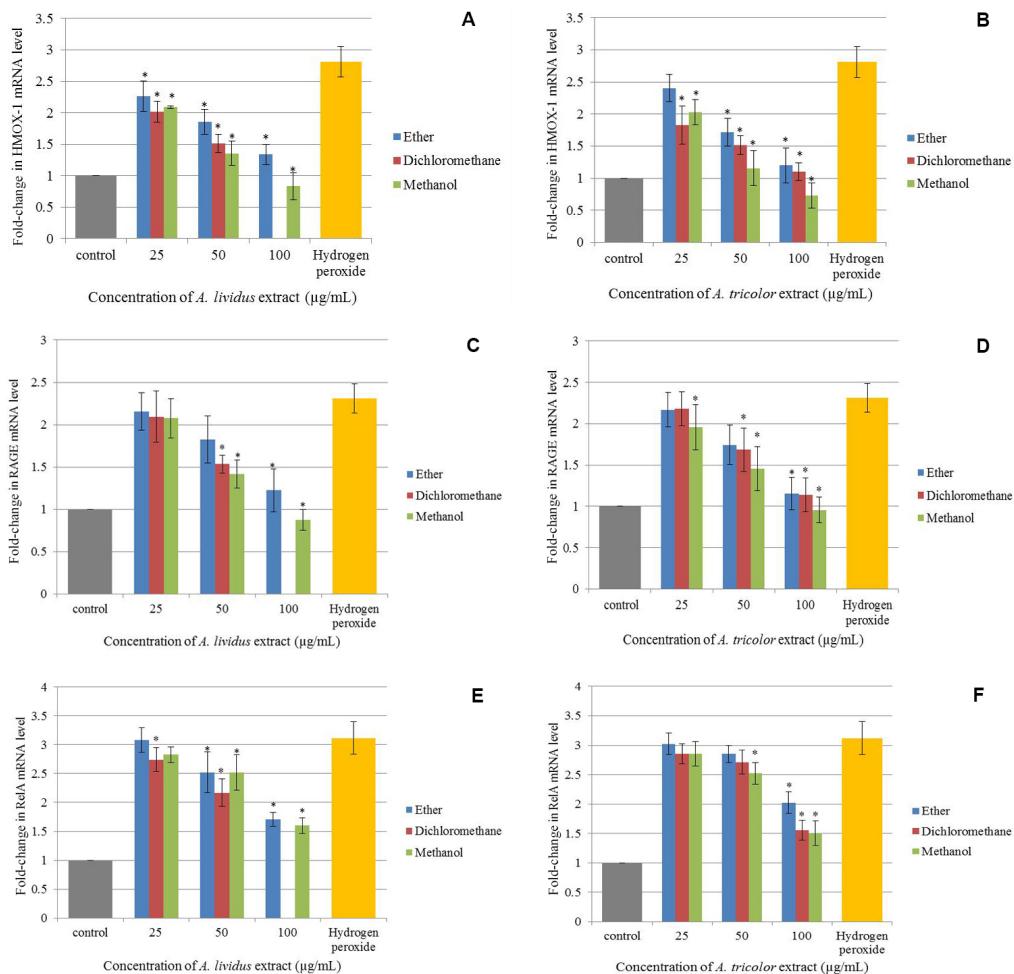


Figure 4. Effect of *Amaranthus lividus* and *Amaranthus tricolor* extracts on *HMOX-1*, *RAGE*, and *RelA* gene expression in SH-SY5Y cells. SH-SY5Y cells were treated with different concentrations of *A. lividus* and *A. tricolor* extracts for 24 h and were then treated with 10 μM H_2O_2 . Gene expression levels of **A.** and **B.** *HMOX-1*, **C.** and **D.** *RAGE*, **E.** and **F.** *RelA* were determined by qPCR analysis. Gene expression was normalized to *GAPDH* expression. Values are reported as means \pm SE, as depicted by vertical bars. All experiments were performed in triplicate (N = 3). *P < 0.05 is considered statistically significant as compared to H_2O_2 -treated cells.

al., 2009; Iqbal et al., 2012; Anitha and Ponbavani, 2013; López-Mejía et al., 2014; Sowjanya et al., 2014). Antioxidants protect living organisms from damage caused by uncontrolled ROS production, which can lead to detrimental effects such as lipid peroxidation, protein damage, and breaking of double-stranded DNA (Ramasamy et al., 2005). As *Amaranthus* plants contain high amounts of natural antioxidants, they may exert protective effects through inhibition of ROS production. In this study, we demonstrated that *A. lividus* and *A. tricolor* extracts can be protective under H_2O_2 -induced oxidative stress. Pretreatment with *A. lividus* and *A. tricolor* extracts significantly decreased cell toxicity and intracellular ROS

production in SH-SY5Y cells. Furthermore, treatment with *A. lividus* and *A. tricolor* extracts significantly decreased gene expression of the *HMOX-1* gene in SH-SY5Y cells. HMOX-1 is normally upregulated during stress, and it is considered one of the most sensitive and reliable indicators of cellular oxidative stress (Poss and Tonegawa, 1997). It was also found to be upregulated in disease models such as Alzheimer's disease (Poss and Tonegawa, 1997). Importantly, our result also showed that *A. lividus* and *A. tricolor* extracts could inhibit gene expression of *RAGE*. Increased ROS level causes activation of 5 major pathways accounting for a diverse group of diseases: polyol pathway flux, increased formation of AGEs, increased expression of the RAGE and its activating ligands, activation of protein kinase C isoforms, and over-activity of the hexosamine pathway (Ramasamy et al., 2005). These pathways further increase production of proinflammatory cytokines and ROS, leading to exacerbated oxidative stress. Previous studies demonstrated that activation of RAGE by RAGE ligands resulted in activation of the RAGE signaling pathway, resulting in increased oxidative stress (Yamagishi et al., 2008; Lee et al., 2010). Therefore, the antioxidant property of *Amaranthus* plants may be associated with inhibition of the RAGE signaling pathway. It is known that activation of the RAGE pathway leads to cell surface signal transductions that are involved in various intracellular pathways including phosphoinositide 3-kinase/AKT, mitogen-activated protein kinase, and NF- κ B (Yan et al., 2010). Additional studies demonstrated that following ligand binding to RAGE, increased oxidative stress further induces overexpression of RAGE, resulting in vicious positive feedback cycles that perpetuate oxidative stress and contribute to neuroinflammation via NF- κ B upregulation (Tóbon-Velasco et al., 2014). The NF- κ B complex plays a fundamental role in the body, and is involved in many pathological conditions. Certain NF- κ B-regulated genes play a major role in regulating ROS levels in the cell. *RelA* is a transcription factor that serves to positively regulate NF- κ B gene expression (Morgan and Liu, 2011). The present study showed that *A. lividus* and *A. tricolor* extracts can effectively decrease NF- κ B activation by suppressing the gene expression of *RelA*. Therefore, *Amaranthus* extracts may play a protective role against oxidative stress by downregulating *RAGE* gene expression via the NF- κ B signaling pathway. However, more research into the exact mechanisms involved in the ROS signaling pathway is needed in order to gain further insight into the beneficial effects of *Amaranthus* extracts.

In conclusion, this study demonstrated for the first time that *Amaranthus* extracts not only protect neuroblastoma cells against oxidative stress, but also decrease oxidative stress by suppressing gene expression of *HMOX-1*, *RAGE*, and *RelA* in SH-SY5Y cells. The neuroprotective effects of *Amaranthus* can be most likely attributed to its antioxidant properties. These findings support the hypothesis that regular consumption of this leafy vegetable may have beneficial effects in reducing oxidative stress-associated risk of neural aging and neurodegenerative diseases.

Conflicts of interest

The authors declare no conflict of interest.

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