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Antioxidant and Anti-inflammatory Protective Properties of Thai Shallot *(Allium ascalonicum* cv. *Chiangmai)* Juice on Human Vascular Endothelial Cell Lines (EA.hy926)[†]

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Abstract

Oxidative stress and inflammation are 2 major contributors to numerous life-threatening disorders, including vascular pathologies. Shallots (*Allium ascalonicum*) are a type of red onion which grows in Southeast Asia. Bulbs of this plant are used both as a food ingredient and in traditional medicine. This study attempted to investigate the possible ways that juice extracted from Thai shallot (*A.ascalonicum* cv. *Chiangmai*) bulbs could be used in the prevention of cardiovascular complications. The antioxidative and anti-inflammatory effects of shallot juice extract (SHE) on human vascular endothelial cells (EA.hy926) were investigated. Cell viability was evaluated by MTT assay, membrane lipid peroxidation by thiobarbituric acid reactive substances assay, intracellular reactive oxygen species (ROS) production by the fluorescent probe 6-carboxy-2'-7'-dichlorofluoresceine, and interleukin-6 (IL-6) released by ELISA. The shallot juice showed extremely low cytotoxicity against EA.hy926 cells, with IC50 of 41.9 and 27.3 mg/ml for 24 h- and 48 h-incubation, respectively. SHE reduced the iron-induced malondialdehyde production in a dose-dependent manner. The extract also demonstrated antioxidant activity as shown by a significant reduction of H_2O_2-induced ROS production at a low concentration (< 200 µg/ml). Furthermore, SHE significantly attenuated the level of IL-6 released during lipopolysaccharide stimulation ($p < 0.05$). It is of interest that the juice extracted from Thai shallot bulbs demonstrated both cellular antioxidant and anti-inflammatory properties in endothelial cell models, combined with a reduction in toxicity. Shallot extract could be considered as a nutraceutical for the prevention or management of vascular diseases as it is related to oxidative stress and inflammation.

Keywords: Shallots, *Allium ascalonicum*, antioxidant activity, anti-inflammatory activity, endothelial cells

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Introduction

The increasing burden of cardiovascular disease (CVD) is a major contributor to the growing public health epidemic in chronic non-communicable diseases (NCD) [1]. The pathogenesis of CVD is strongly related to oxidative stress [2], which is caused by an imbalance in the production of the reactive oxygen species (ROS) and the abilities of the endogenous antioxidant system. The mechanism involves oxidative damage to endothelial cells, accelerating inflammation and triggering endothelial dysfunction, factors affecting the initiation and progression of atherosclerosis [3]. Therefore, it is conceivable that substances with anti-oxidative and anti-inflammatory properties, or showing properties protective of the vascular endothelium, will be beneficial in the management of CVD [1].

Conventional medicine for treatment of CVD is generally expensive, and beyond the reach of people in many developing countries. The search for affordable therapies has led to possible natural alternatives [4]. Plants are rich in secondary metabolites, including phenolics, flavonoids and other compounds that contain antioxidants, which are recognized for their therapeutic properties. In addition, factors such as availability, accessibility, affordability and the natural safety of medicinal plants have led to an increase in demand and usage [1].

Allium plants are an important dietary component, and there has been a long-held belief in their health-promoting properties [5]. Shallots (*Allium ascalonicum*) are a type of red onion, grown in Southeast Asia. Bulbs of this plant are used as a condiment in Asian cuisine and also serve as an ingredient in traditional medicine to relieve fevers, flatulence and infections.

Shallots are a rich source of vitamins (A, B6), organo-sulfur compounds (allin, allicin), as well as flavonoids and phenolic compounds (quercetin, kamferal) [6]. These compounds are recognized for their biological properties, including antibacterial, antivirus, anti-diabetic, antioxidant, and anti-inflammation uses [7]. Thus, shallot extract received a great deal of attention as a remedy for attenuation of free radical damage and for treatment of various oxidative-related disorders. Unfortunately, although shallots are one of the most promising medicinal plants, only few studies of the Thai shallot had been carried out. In Thailand, 2 main shallot varieties have been cultivated: *Chiangmai* and *Srisaket*. These have been named for their major agricultural areas. The *Chiangmai* variety shallots have an orange-yellow skin and pale yellow-colored flesh. When compared with the *Srisaket* variety, of *Chiangmai* variety bulbs tend to be more tapered and smaller, which about 1 - 2 inches in diameter. The flavor is also milder and more like an onion.

The aim of this study was to investigate the juice extracted from Thai shallot bulbs (*A. ascalonicum* cv. *Chiangmai*) to determine their potential for the prevention of cardiovascular complications. The antioxidative and anti-inflammatory effects of shallot juice extract (SHE) were evaluated in human vascular endothelial cell lines (EA.hy926) subjected to oxidative stress.

Materials and methods

Reagents

Reagents for cell cultures, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin/EDTA were purchased from Gibco (New York, USA). Aluminum chloride, ethanol, and sodium carbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Folin-Ciocalteu reagent, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), potassium persulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonate) diammonium salts (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), gallic acid, quercetin standards and other chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All reagents and solvents used in the assay procedures were of analytical, HPLC or cell culture grade with the most suitable level of purity.

Plant material and preparation of shallot juice extract

Pesticide-free bulbs of *A. ascalonicum* cv. *Chiangmai* were collected from a local field in Chiangmai, Thailand (GPS coordinates: 18°38'39.9"N 98°49'34.3"E) during March-April 2017. The plant

materials were identified by Dr. Prachaya Srisanga, Herbarium Curator at The Botanical Garden Organization, Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai and voucher specimens was deposited in the Queen Sirikit Botanic Garden Herbarium, Chiang Mai, with voucher number SK-SH-CM01. The bulbs were peeled and the juice was obtained by using an extractor. The fresh shallot juice was then filtrated through sterile gauze, centrifuged at 3,000 rpm at 4 °C for 15 min and re-filtered through filter paper Whatman No.1. The filtrate obtained was then immediately subjected to a freeze dryer. Powder of shallot juice extract (SHE) was kept at −20 °C until further analysis could be performed. Before being used, SHE was weighed and re-suspended in HPLC grade deionized (DI) water (for chemical analysis) or sterile phosphate buffer saline (for cell culture tests).

Determination of total phenolic content

The SHE was re-suspended in DI water and total phenolic content was determined through a Folin-Ciocalteu assay as previously described [8]. Briefly, 20 µl of standard or sample solution was mixed with 1.58 ml water, and 100 µl of 2N Folin-Ciocalteu reagent. After waiting for 5 min, 300 µl of saturated sodium carbonate solution was added. The mixtures were incubated at 40 °C for 30 min, and then their absorbance was measured at 765 nm against the reagent blank. The calibration curve was prepared using various concentrations (50 - 500 mg/ml) of gallic acid. The results were expressed as milligram gallic acid equivalent per gram fresh weight (mg GE/g FW).

Determination of total flavonoid content

The total flavonoid content of the extracts was measured using a colorimetric method developed by Zhishen *et al*. [9]. A volume of 500 µl of 10 % SHE solution was mixed with 150 µl of a 5 % $NaNO_2$ solution. After 5 minutes, 150 µl of 10 % $AlCl_3$ solution was added and allowed to stand for 60 minutes. Then, 1 ml of 1M NaOH solution was added, followed by the addition of DI water to bring the final volume to 3 ml. The mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance against reagent blank was determined spectrophotometrically at 510 nm. The results were expressed as milligram quercetin equivalent per gram fresh weight (mg QE/g FW).

Determination of total antioxidant capacity

Trolox Equivalent Antioxidant Capacity (TEAC) was determined using an ABTS radical cation decolorization assay according to a slight modification of a method described by Re et al. [10]. Briefly, the pre-formed radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\circ+}$) was generated by oxidation of 7 mM ABTS solution with 2.45 mM potassium persulfate solution in the dark, at room temperature for 12 h. A working $ABTS^{\circ+}$ solution was obtained by adjusting the absorbance at 734 nm to 0.700±0.02 with absolute ethanol. A sample of the SHE solution was added to the $ABTS^{\circ+}$ solution in a ratio of 1:10 *v/v* and the absorbance was measured at 734 nm after exactly 3 min. Trolox, a water-soluble tocopherol derivative, was used as a reference compound. The results were expressed as milligram trolox equivalent per gram fresh weight (mg TE/g FW).

Cell culture

The permanent human vascular endothelial cell line EA.hy926 (ATCC® CRL 2922™) was originally derived from a human umbilical vein obtained from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution at 37 °C in a humidified atmosphere of 5 % CO_2. When the cells reached 70 - 80 % confluence cells were harvested and plated, either for subsequent passages or for the treatments.

The SHE was analyzed for possible cytotoxicity by using a cell viability test. To study the role of SHE in prevention of oxidative stress-related endothelial dysfunction, chemical compounds were used to produce exogenous ROS which invaded the cell, and then the parameters including membrane lipid peroxidation, intracellular ROS production and inflammatory cytokine released were determined.

Cell viability test

Changes in cell survival in response to SHE were determined using an MTT assay. In brief, cells were seeded overnight into a 96-well microplate at a density of 5×10^3 cells/well. The day after, the medium was changed into a fresh complete DMEM medium containing various concentrations of SHE (25 - 500 mg/ml). The same volume of PBS was used as the negative control (0 µg/ml). After 24 or 48 h of treatments, the cells were washed twice with PBS and harvested. Then, 20 µl MTT dye (5 mg/mL in PBS) was added, and the incubation continued at 37 °C for 4 h. Finally, the medium was removed and the MTT-formazan crystal was dissolved in 100 µl DMSO. The absorbance wash was measured at λ540/630 nm with a microplate reader (STECTROstar Nano, BMG LABTECH, Germany).

H_2O_2-induced intracellular ROS production

The antioxidative effects of SHE on intracellular oxidative stress were investigated in a model of H_2O_2-induced intracellular ROS production, as measured by the DCFH-DA assay. EA.hy926 cells were seeded at a density of 1×10^4 cells/well onto a 96-well culture plate and incubated with various concentrations of SHE (25, 50, 100, 200, 500 µg/ml) or 200 µg/ml vitamin E, for 24 h at 37 °C, 5 % CO_2. Afterwards, levels of intracellular ROS were measured using fluorescence probe dichlorofluorescin diacetate (DCFH-DA) in presence of H_2O_2 [11]. In brief, following removal of the culture medium, the treated cells were washed twice with PBS. Ten µM of DCFH-DA in complete media was then added and incubated in the dark at 37 °C for 30 min. After the excess dye was washed away, 125 µM H_2O_2 was added to the cells to stimulate ROS production and incubated again for further 30 min. Fluorescence intensity (FI) of dichlorofluorescein (DCF), directly proportional to the amount of intracellular ROS, was detected using fluorescent plate reader with $\lambda_{ex/em}$ 485/530 nm [12].

FeAS-induced lipid peroxidation

The effects of SHE on the prevention of oxidative damage to lipid membranes were investigated. Ferrous ion from ferrous ammonium sulfate (FeAS) was used to catalyze a Haber-Weiss and Fenton reaction, which subsequently caused an ROS-induced lipid peroxidation. Malondialdehyde (MDA), an end product of lipid peroxidation, was measured for its response to the extent of oxidative membrane damage. Briefly, cells were seeded in a density of 1×10^6 cell/well in a 6-well culture plate and incubated with different concentrations of SHE (50 - 500 µg/ml) or 200 µg/ml vitamin E, in the presence of 5 mM ferrous ammonium sulfate for 24 h at 37 °C, 5 % CO_2. At the end of the incubation period, cells were collected using a cell scraper and washed with PBS. Cells were homogenized and centrifuged for pellets, whose levels of MDA were measured by thiobarbituric acid reactive substances (TBARS) assay [13]. Cell homogenate (prepared in 0.2 ml PBS) was mixed with 0.3 ml of 1 % phosphoric acid and 0.1 ml of 0.67 % TBA in a test tube and heated in boiling water for 60 min with the tube sealed. After cooling to room temperature, a solvent extraction of MDA was created by the addition of 0.4 ml *n*-butanol. The absorbance of the clear solution, measuring MDA concentration, was calculated at λ535 nm.

Lipopolysaccharide-induced cytokine release

The anti-inflammatory activity of SHE in EA.hy926 cells was investigated by measuring the release of inflammatory cykokine, interleukin-6 (IL-6). Cells were seeded in a density of 1.5×10^6 cell/well in a 6-well culture plate and incubated with different concentrations of SHE (50 - 500 µg/ml) or 10 µg/ml of an anti-inflammatory drug, dexamethasone in the presence of 1 µg/ml lipopolysaccharide (LPS) for 24 h at 37 °C, 5 % CO_2. Levels of IL-6 release into the culture media were determined by using a Human IL-6 ELISA Kit (ab46027) from Abcam (USA), following manufacturer's protocol.

Statistical analysis

All measurements were given as mean ± standard derivation from triplicate samples of three independent experiments. Overall differences between the treatment groups were determined by using a one-way ANOVA of SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered as statistically significant.

Results and discussion

Oxidative stress and inflammation are 2 major contributors to numerous life-threatening disorders, including vascular pathologies. Increases in intracellular ROS induces oxidative stress, which then triggers cellular inflammatory responses and causes atherosclerosis of the endothelium [14,15].

Medicinal plants which display biological antioxidant activity have been shown to benefit the endothelium. In this study, the protective effects of SHE on prevention of oxidative stress-mediated endothelial dysfunction were investigated.

Total phenolic, total flavonoid content and total antioxidant capacity of shallot juice extract

Extract of juice from shallot (*A. ascalonicum* cv. *Chiangmai*) bulbs giving a dry pale yellow powder with a yield of 6.35±0.35 % fresh weight (FW) basis. The total amounts of phenolic and flavonoid contents in the extract were 1,658.17±16.70 mg GAE/g FW and 98.54±2.13 µg QE/g FW, respectively. The anti-oxidative effects of the SHE were tested in a cell-free system using a TEAC assay based on the ABTS decolorization method. The total antioxidant capacity of the extract, determined by its ability to scavenge $ABTS^{\bullet+}$ radicals, was found to be 246.99±2.24 µg TE/g FW.

In addition, fresh juice extraction was demonstrated to be a suitable method for getting shallot extract with acceptable yields and strong antioxidant activity [16,17].

Cytotoxicity test

Treatment of the cells by SHE resulted in a dose-dependent decrease of cell viability, with an IC50 of 41.9 and 27.3 mg/ml for 24 h and 48 h-incubation times, respectively (**Figure 1**). However, at doses up to 200 µg/ml the SHE did not have significant effects on EA.hy926 cell viability (data not shown).

According to the U.S. National Cancer Institute (NCI), the criteria of cytotoxicity for the crude extract is IC50 < 20 µg/ml [18]. Hence, SHE was noticeable not toxic to the cells.

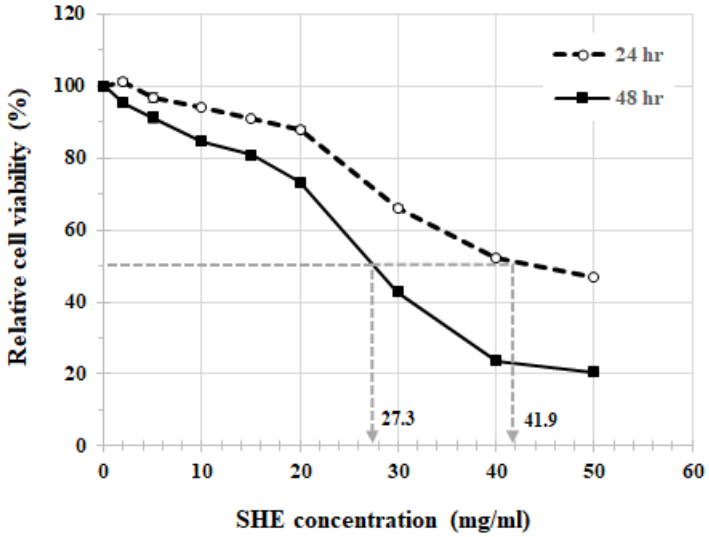

Figure 1 Growth-inhibitory effect of shallot juice extract in EA.hy926 cells.

Protective effects of SHE against iron-induced lipid peroxidation

Iron is a transition metal which is capable of accelerating ROS formation by catalyzing a Fenton reaction. The promotion of a hydroxyl radical formation results in a reduction of cellular antioxidants, and oxidative damage to biomolecules i.e, protein degradation, lipid peroxidation and DNA modification.

Endothelial EA.hy926 cells exposed to 5 mM ferrous ammonium sulfate (FeAS) resulted in a significantly increased level of malondialdehyde (MDA), an end-product of membrane lipid peroxidation. Treatment of the cells with SHE significantly reduced iron-induced MDA production in a dose-dependent fashion (**Figure 2**). The anti-oxidative effects of SHE on the protection of cellular membranes was found to be comparable to a known antioxidant, vitamin E. The ability of SHE to inhibit iron-induced lipid peroxidation may be based on its iron-binding capacity or peroxyl radical scavenging properties. Further studies should be performed to clarify these findings.

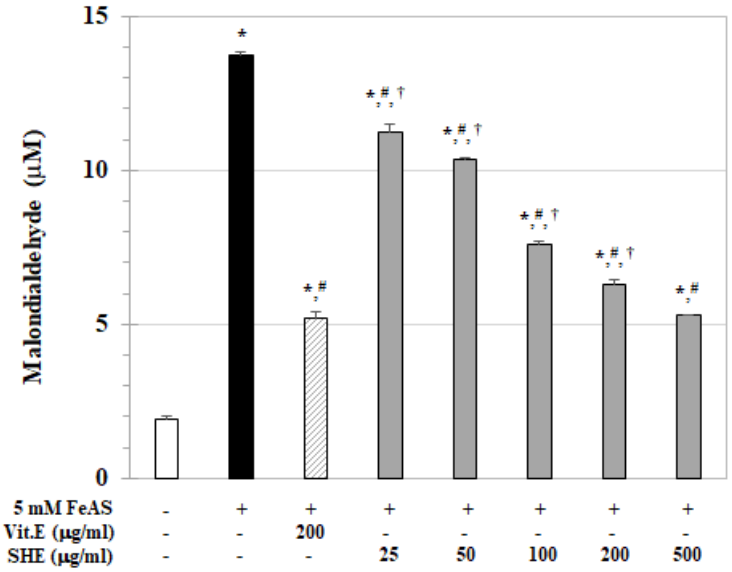

Figure 2. Levels of malondialdehyde in cultured endothelial cells in response to ferrous iron-induced lipid peroxidation. $^{*}p < 0.05$ compared to basal control (white bar), $^{\#}p < 0.05$ compared to negative control (black bar). $^{\dagger}p < 0.05$ compared to positive control (striped bar).

Antioxidative property of SHE protecting against H_2O_2-induced oxidative damage

To evaluate the intracellular anti-oxidative properties of SHE in endothelial cells, ROS production was induced by an explosion of H_2O_2 and was measured based on its reaction to DCFH-DA. DCFH-DA is the most widely used probe sensitive for the detection of intracellular H_2O_2 and oxidative stress [19]. The probe is able to penetrate through cell membranes and is hydrolyzed by cellular esterase to produce a more hydrophilic non-fluorescent dichlorofluorescin (DCFH). In the presence of intracellular ROS, rapid oxidation of DCFH results in a proportionate formation of a green fluorescent product, dichlorofluorescein (DCF), whose intensity can be monitored by fluorescence-based techniques.

Results shown in **Figure 3** prove that peroxyl radicals (from H_2O_2) could dramatically induce oxidation of DCFH to DCF in human endothelial cells. In the presence of 200 μg/ml vitamin E, induction of ROS production was statistically inhibited ($p < 0.05$). Likewise, in a pre-treatment of SHE, at a dose lower than 200 μg/ml, prior H_2O_2 induction could significantly reduce intracellular ROS production. In contrast, when SHE was used in a higher concentration, it failed to significantly reduce the levels of ROS in the cell. This may suggest that in certain conditions, SHE exhibits an anti-oxidative effect with a low dose, and a pro-oxidative ability with a high dose.

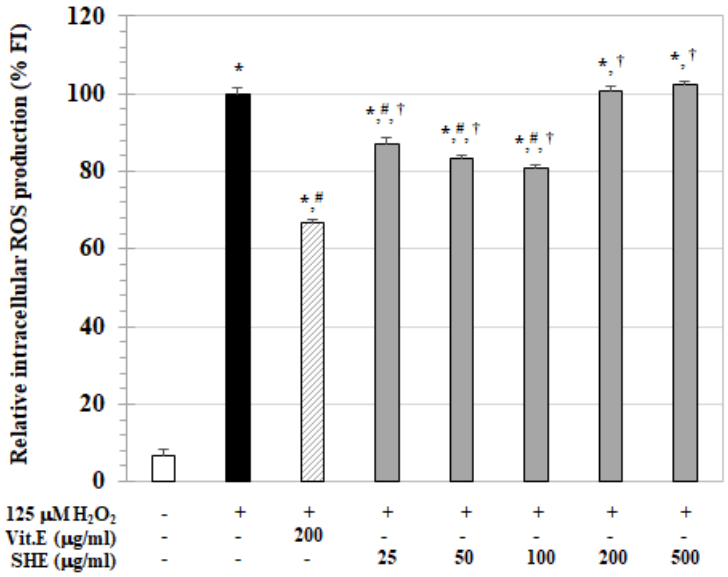

Figure 3 Relative intracellular ROS production in EA.hy926 cell during H_2O_2 exposure. $^{*}p < 0.05$ compared to basal control (white bar), $^{\#}p < 0.05$ compared to negative control (black bar). $^{\dagger}p < 0.05$ compared to positive control (striped bar).

Anti-inflammatory effect of SHE in LPS-induced endothelial cell line

Chronic inflammation and endothelial cell activation play crucial roles in vascular atherogenesis, and were also found to be a high-risk factors for development of cardiovascular disease [20]. During the inflammation process, a variety of inflammatory cytokines are released and aggravate endothelial dysfunction.

In this study, lipopolysaccharide (LPS) was used to accelerate inflammation and the release of inflammatory cytokines. Levels of interleukin-6 (IL-6) released into culture media were measured as a representative parameter of vascular endothelial injury.

It was observed that SHE can inhibit the elevation of IL-6 levels induced by LPS in a dose-dependent manner. The result indicated an anti-inflammatory activity of SHE in endothelial cells. This ability, however, was minor compared with synthetic anti-inflammatory drugs like dexamethasone (**Figure 4**).

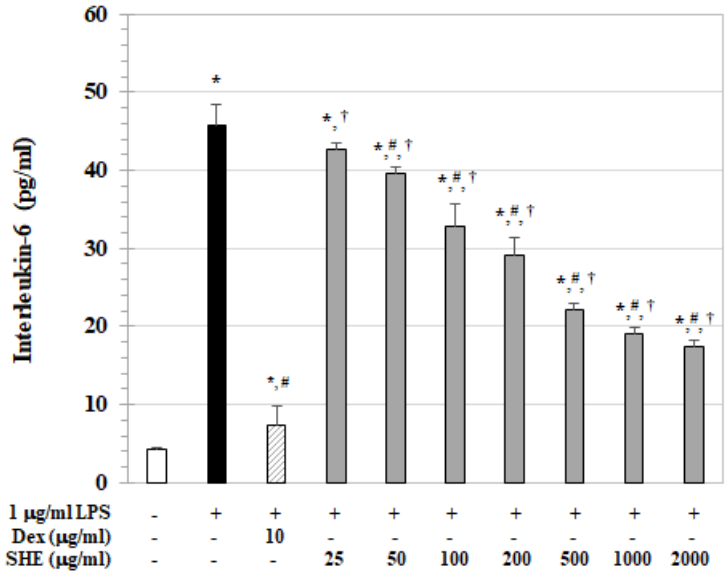

Figure 4 Levels of interleukin-6 release in response to LPS-induced endothelial inflammation. $^{*}p < 0.05$ compared to basal control (white bar), $^{\#}p < 0.05$ compared to negative control (black bar). $^{\dagger}p < 0.05$ compared to positive control (striped bar).

Taken together, our findings suggest that SHE may be a potential candidate for the management of various atherosclerotic disorders. Active substances present in SHE, such as phenolics and flavonoids, may contribute to a great variety of health-related physiological activities, including a reduced risk of cardiovascular diseases. This is partly due to its ability to scavenge free radicals, prevent membrane lipid peroxidation and inhibit inflammation, thereby protecting endothelial cells from oxidative damage. The cellular antioxidant activity of the extract in endothelial cells is therapeutically important. ROS are produced at all layers of the vascular wall, especially at the endothelium [21] and responsible for vascular endothelial injury. Consequently, the prevention of oxidative stress-induced endothelial dysfunction has become a major therapeutic target in the management of cardiovascular complications [22].

Conclusions

It is of interest that juice extracted from Thai shallot bulbs causes a substantial level of antioxidant and anti-inflammatory activities in normal endothelial cells, combined with considerable non-toxicity in terms of viability during the first 48 hours of culture. Shallot extract could be considered a promising regimen for management of diseases related to oxidative stress and inflammation.

Acknowledgements

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