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Assessment of Antioxidative Properties of Aqueous Leaf Extract of *Anacardium occidentale* L. On Human Umbilical Vein Endothelial Cells

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ABSTRACT

The aim of this study was to evaluate the antioxidant potential of aqueous leaf extract of *Anacardium occidentale* (AOE) against hydrogen peroxide (H_2O_2)-induced oxidation on human umbilical vein endothelial cells (HUVECs). HUVECs were exposed with various concentrations of H_2O_2 (0-700 μM) and it was observed that 250 μM of H_2O_2 reduced cell viability by 50% (inhibitory concentration, IC_{50}) as denoted by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Using the above concentration as the positive control, the cells were pretreated with AOE at various concentrations (50-700 μg mL^{-1}) for 30 min, followed by 24 h incubation with H_2O_2 (250 μM). The AOE (100-300 μg mL^{-1}) was found to protect cellular damage and prevent microsomal lipid peroxidation in H_2O_2-induced HUVEC indicated with low MDA levels. Furthermore, AOE at similar concentrations may directly act on H_2O_2 as indicated with reduction of Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) activities. Overall, these data suggested that AOE possessed antioxidative properties against H_2O_2-induced oxidation on HUVECs and improved the endogenous antioxidant enzymes activity *in vitro*.

Key words: *Anacardium occidentale*, human umbilical vein endothelial cells, oxidative stress, malondialdehyde, antioxidant enzymes

INTRODUCTION

Oxidative stress plays an important role in the pathogenesis of various cardiovascular diseases including atherosclerosis (Ross, 1999). At high concentrations, free radicals or Reactive Oxygen Species (ROS) may oxidize cellular structures and other biological components including nucleic acids, lipids and proteins leading to cellular deterioration and cell death (Penna *et al*., 2009). Reports from various epidemiological study highlighted that ROS such as superoxide anion ($O^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are closely involved in the pathogenesis of various diseases such as neurodegenerative diseases associated with aging, cancer, cardiovascular diseases and atherosclerosis (Jung *et al*., 2008). On the other hand, free radicals are neutralised

by an elaborate antioxidant defence system consisting of enzymatic antioxidants such as Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) and numerous non-enzymatic antioxidants including vitamins A, E and C, glutathione, carotenoids and flavonoids. These antioxidants are able to scavenge free radicals and interrupt lipid peroxidation thus minimizing the effect of oxidative stress (Estany *et al*., 2007; Ihsan *et al*., 2011).

Anacardium occidentale commonly called cashew is a well known traditional medicine within Asia countries such as India, Malaysia and Indonesia (Razali *et al*., 2008). The plant has been traditionally used to treat various diseases including diabetes, diarrhea, malaria and yellow fever (Akinpelu, 2001; Concalves *et al*., 2005). Investigations of the phytochemical components of the plant have revealed the presence of various compounds such as flavonoids, glycosides and glucose which possessed high antioxidant activities (Konan and Bacchi, 2007; Razali *et al*., 2008).

Although, many antioxidant activities and phenolic content of this plant have been previously reported, the focus of the studies are mainly on the nuts and stem barks (Kornsteiner *et al*., 2006; Kubo *et al*., 2006). The leaves are commonly consumed as salads but limited information on the antioxidant activities and its potential effect on free radical mediated diseases had been reported. This study was therefore, undertaken to evaluate the antioxidant property of aqueous leaf extract of *A. occidentale* by determining its ability to reduce the lipid peroxidation product; Malondialdehyde (MDA) and altering the activities of antioxidant enzymes namely Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) which act as oxidative stress markers in hydrogen peroxide (H_2O_2)-induced oxidation on human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Preparation of 10% *Anacardium occidentale* aqueous extract: The fresh leaves of *A. occidentale* were sourced from Forest Research Institutes of Malaysia (FRIM) after being identified and authenticated by a plant taxonomist. A voucher specimen was deposited in the Institute of Bioscience, UPM (SK233). The leaves were cut into small pieces, dried and pulverized. One hundred gram of the powdered leaves were soaked in distilled water and make the final volume to 1000 mL followed by incubation in shaking water bath at 60°C for 6 h. Once filtered, it was freeze dried and kept at -20°C until used.

Cell culture: Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Rockvilled, MD, USA). The cells were maintained in a T-25 flasks (Nunc, Roskilde, Denmark) containing M 200 medium and Low Serum Growth Supplement (LSGS) (Cascade Biologies Inc, Sweden) in a humidified atmosphere of air with 5% CO_2 at 37°C and routinely subcultured in every two days. Uniform monolayers from the primary culture were formed after 6–8 days and TE (trypsin/EDTA) solution (a sterile, phosphate-buffered saline solution containing 0.025% trypsin and 0.01% EDTA, with pH of 7.2 at room temperature) was used to harvest cells. Only 3rd-5th passage were used in the experiments.

Cell viability: In order to obtain the inhibitory concentration of H_2O_2 or toxicity potential of AOE on HUVECs, firstly the cells (1×10^6 cells per well) were seeded in 96-well plates and incubated for 24 h. Following incubation, H_2O_2 or AOE at concentration of 100, 200, 300, 400, 600 and 700 μM is added and incubated for the subsequent 24 h. In addition, the protective effect of AOE on H_2O_2-oxidative cell damage was examined by pretreating the cells with AOE (0-1000 μg mL^{-1}) for 30 min

and concomitantly exposed to H_2O_2 (250 μM) for further 24 h. Cell viability was assessed using the MTT assay as previously described (Takahashi *et al.*, 2002). In brief, the cultures were washed with PBS, 20 μL of 5 mg mL^{-1} of MTT solution was added and the cells were incubated for 4 h. After that, the media were removed, 50 μL of Dimethyl Sulfoxide (DMSO) was added to each well. Absorbance at 570 nm was determined by a microplate ELISA reader (Grodig, Austria). The percent of cell viability was calculated according to the formula below:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of experimental group}}{\text{Absorbance of blank control group}} \times 100\%$$

Experimental protocol: At the initial time of the experiment, HUVECs (1×10^6 cells per well) were seeded in 96 well plate and incubated for 24 h in complete medium. After the incubation period, the initial medium was replaced with the fresh ones followed by pretreatment with AOE at various concentrations (100, 180, 250 and 300 μg mL^{-1}) for 30 min and subsequent 24 h treatment with H_2O_2 (the IC_{50} concentration). Positive Control (PC) was denoted as cells treated with H_2O_2 alone without the extracts whereas Negative Control (NC) was cells with only the culture medium. After the incubation period, the cells were homogenized. The cell lysate obtained was used for the determination of biochemical assay.

The MDA level: Lipid peroxidation was assayed by determining the production rate of TBARS and was expressed as MDA equivalents (Ohkawa *et al.*, 1979). Its absorbance was determined at 532 nm spectrophotometrically (Shimadzu, Japan). The level of MDA in sample was determined from the 1,1,3,3-Tetraethoxypropane (TEP) serial dilution standard graph and the MDA level was expressed as nmol MDA mg^{-1} protein. Protein analysis was governed by Lowry method as previously described (Lowry *et al.*, 1951).

The SOD, CAT and GPx activities: The activities of SOD were measured according to the method of Beyer and Fridovich (1987). CAT activities were determined as previously described (Aebi, 1984). The activities of GPx were assayed as per previous protocol (Lawrence and Burk, 1976).

Statistical analysis: Data were expressed as Mean±Standard deviation of triplicate samples. Statistical analysis was performed by one-way ANOVA with Tukey's *post hoc* multiple group comparison using Statistical Package for Social Sciences software (SPSS 12.0.1, Chicago, IL, USA). $p<0.01$ and $p<0.05$ were considered significant for all tests.

RESULTS

Cell viability: Figure 1-3 showed the percentage of viable cells upon exposure to H_2O_2 or AOE at various concentrations. H_2O_2 was observed to cause 50% cell death at concentration of 250 μM after 24 h incubation (Fig. 1) and that concentration was used as positive control for the subsequent experiment. Conversely, incubation of HUVEC with AOE at all concentration range for 24 h was shown to be non toxic as no cell death was observed. Hence, no IC_{50} value for AOE was obtained (Fig. 2). In addition, the protective effect of AOE on H_2O_2-oxidative cell damage was found to be effective at 180 μg mL^{-1} (Fig. 3).

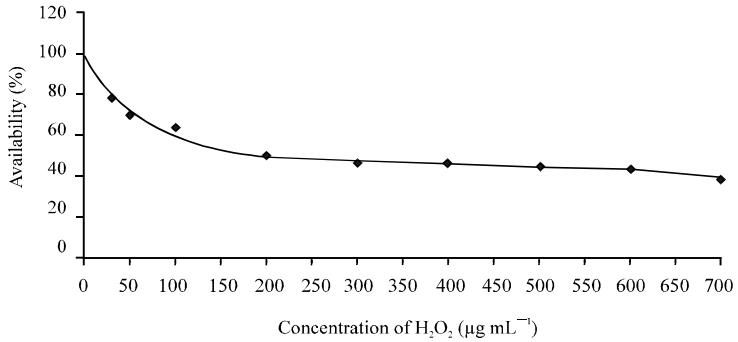

Fig. 1: The percentage of human umbilical vein endothelial cells (HUVEC) viability treated with H_2O_2

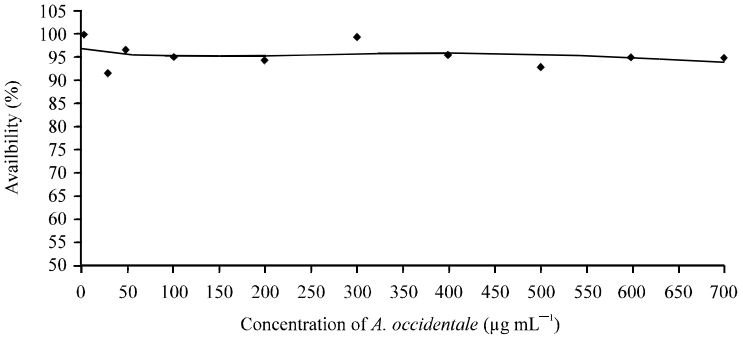

Fig. 2: The percentage of human umbilical vein endothelial cells viability treated with AOE

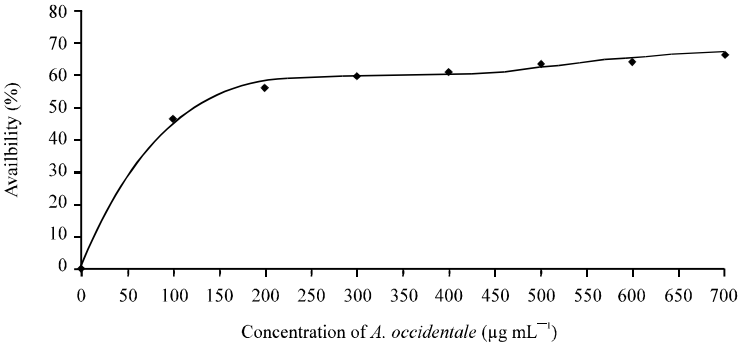

Fig. 3: The effect of AOE on H_2O_2-induced human umbilical vein endothelial cells

The MDA level: As shown in Fig. 4, H_2O_2 clearly causes cellular injury and elevated process of lipid peroxidation indicated with a significant increase of MDA concentration in three fold increment compared to the NC group ($p<0.01$). However, AOE demonstrated a protective effect against H_2O_2-induced oxidative stress and exhibit antioxidative properties with remarkable 3 fold reduction of MDA concentration in all treatment groups compared to the PC group ($p<0.01$).

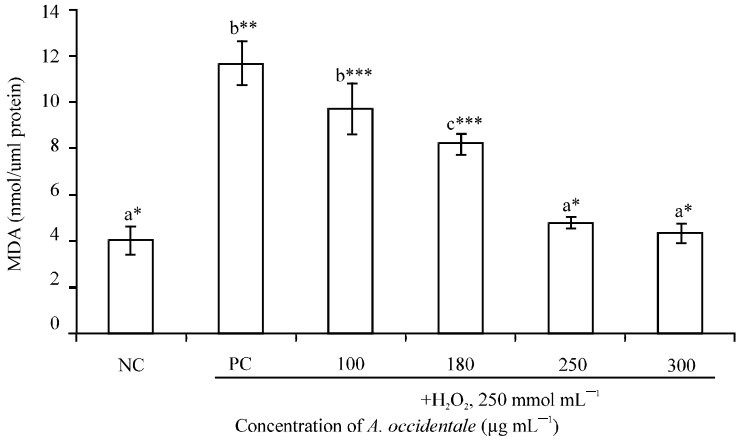

Fig. 4: The effect of AOE on the level of MDA in H_2O_2-induced human umbilical vein endothelial cells (HUVEC). Data were expressed as Mean±SD, Values with different letter were significantly different between samples ($p<0.05$). Values with different asterisk number were significantly different between samples ($p<0.01$)

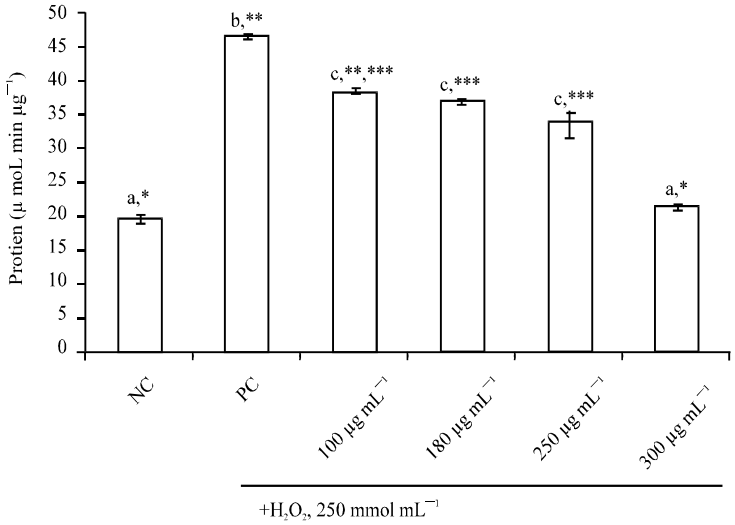

Fig. 5: The effect of AOE on the activity of SOD in H_2O_2-induced human umbilical vein endothelial cells (HUVEC), Data were expressed as Mean±SD, Values with different letter were significantly different between samples ($p<0.05$). Values with different asterisk number were significantly different between samples ($p<0.01$)

The SOD, CAT and GPx activities: The positive control group showed a remarkable increased in the activities of SOD, CAT and GPx , respectively compared to the NC group ($p<0.05$) (Fig. 5-7). Conversely, groups pretreated with AOE at all concentration demonstrated a reduction of SOD, CAT and GPx activities compared to the PC group ($p<0.05$) and the reduction was observed to be in a dose dependent manner. The SOD reduction by AOE treatment was observed effectively at minimal concentration of 100 µg mL^{-1} (Fig. 5) ($p<0.05$) followed by 180, 250 and 300 µg mL^{-1} , respectively. Similar results were obtained for the analysis of CAT and GPx, where the activity of

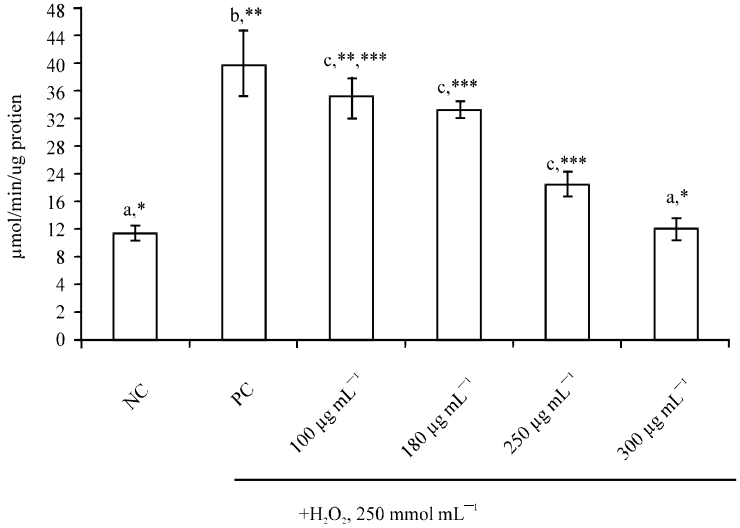

Fig. 6: The effect of AOE on the activity of CAT in H_2O_2-induced human umbilical vein endothelial cells (HUVEC). Data were expressed as Mean±SD Values with different letter were significantly different between samples ($p<0.05$). Values with different asterisk number (*vs.**) were significantly different between samples ($p<0.01$)

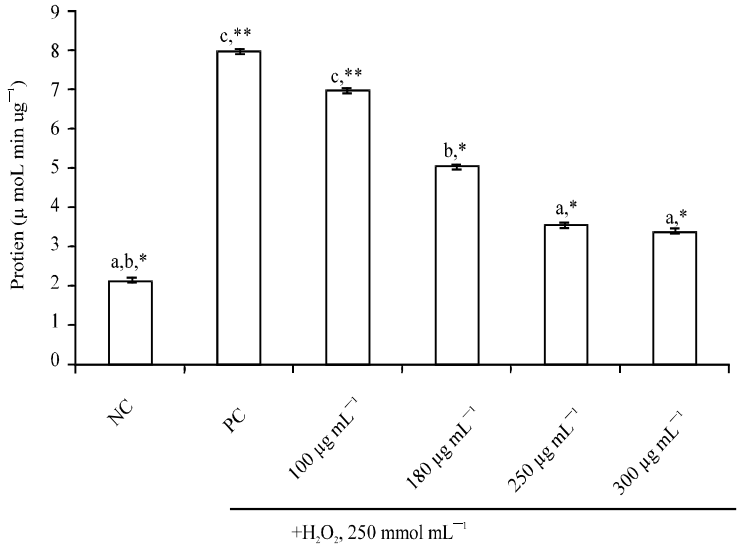

Fig. 7: The effect of AOE on the activity of GPx in H_2O_2-induced human umbilical vein endothelial cells (HUVEC). Data were expressed as Mean±SD Values with different letter were significantly different between samples ($p<0.05$). Values with different asterisk number were significantly different between samples ($p<0.01$)

these two enzymes in cells pretreated with AOE were found to be reduced at minimal dose of 100 µg mL^{-1}. Furthermore, the treatment of AOE at dose of 300 µg mL^{-1} in all groups demonstrates the lowest SOD, CAT and GPx activities significantly compared to the PC group ($p<0.01$) (Fig. 6-7). Interestingly, the activity of SOD, CAT and GPx in the NC group was found to be comparable with groups treated with AOE at dosage of 300 µg mL^{-1} hence, suggesting the protective effect of AOE against lipid peroxidation induced by H_2O_2 on HUVECs.

DISCUSSION

Oxidative stress, arising as a result of imbalance between free radicals generation and endogenous antioxidants defence in body plays a key role in the initiation and progression of many diseases including inflammation, rheumatoid arthritis, cancer, cardiovascular diseases and atherosclerosis (Halliwell and Gutteridge, 1989). Hydrogen peroxide (H_2O_2) is described as non-free radical but its reactivity is very strong resembling Reactive Oxygen Species (ROS) that may diffuse inside and outside the cells which then acts as precursor of oxidation via reaction with metal ions or heme peroxides (Penna *et al*., 2009). H_2O_2 is generated by multiple intracellular sources including mitochondrial and other electron transport chains, oxidase enzymes (e.g., xanthine oxidase) and uncoupled Nitrogen Oxygen Species (NOS) (Halliwell and Gutteridge, 1989). The result from this experiment demonstrated that exposure of H_2O_2 (250 μM) to HUVECs could cause 50% cell death and this particular concentration was used as positive control for the subsequent experiments. The IC_{50} of H_2O_2 that was determined from this experiment was near to the concentration of H_2O_2 obtained from the metabolic control analysis (Ihsan *et al*., 2012). Furthermore, much evidences revealed that H_2O_2 can cause endothelial cell injury by inducing mitochondrial dysfunction and cell death (Cianchetti *et al*., 2007; Hafizah *et al*., 2010).

Dietary supplements consisting of antioxidants such as flavonoids and vitamins are considered to effectively protect body cells from the attack by oxidative stress and therefore, preserve human body in general health (Sies *et al*., 2005; Rahman *et al*., 2006). There is a growing interest in traditional Asian medicines as they are pharmacologically potent with low or no toxicity and rarely produce complications. Many phenolic compounds derived from these natural plants have been reported to possess potent antioxidant activity against cancer, bacteria, virus, inflammation and thrombosis (Seyoum *et al*., 2006). In this present study, AOE was shown to be non toxic to HUVECs even to the highest concentration (700 μg mL^{-1}) as no IC_{50} value was obtained. In addition, AOE was found to protect HUVECs from H_2O_2 oxidation starting from concentration of 180 μg mL^{-1} where the cell viability was increased.

Malondialdehyde (MDA) is the most abundant product of polyunsaturated lipid peroxidation induced by excessive ROS. In this study, H_2O_2 markedly increased the MDA level in HUVECs. The result was consistent with previous studies showing an increase in the MDA level following addition of H_2O_2 and other ROS precursors like carbon tetrachloride (CCl_4) and Azoxymethane (AOM) in various types of cells (Ashokkumar and Sudhandiran, 2008; Hafizah *et al*., 2010). In contrast, cells pretreated with AOE prevent cell apoptosis and reduce formation of MDA in HUVECs. This result suggested that AOE protected membrane cell damage from excessive ROS. Thus, AOE is speculated to exhibit antioxidative effects against membrane lipid peroxidation damage by its ability in penetrating the lipid bilayer and interacting with ROS within the cell membrane. This is in agreement with previous studies demonstrating other phenolic plants such as *Piper sarmentosum* and *Silybum marianum* and also luteolin supplementation that reduce the MDA concentration in ROS-induced cells (Wang *et al*., 2005; Hafizah *et al*., 2010).

Anacardium occidentale contained high total phenolic content that likely contributed to its high antioxidant activities, thus suggesting AOE as a source of natural antioxidants (Razali *et al*., 2008). The phenolic compounds are considered effective antioxidants with their capability to act as free radical scavengers and inhibitors of lipid peroxidation because of their metal-chelating and radical scavenging properties (Bors and Saran, 1987; Halliwell and Gutteridge, 1989). Based on previous study, the shoot of *A. occidentale* was able to inhibit copper-induced LDL oxidation (Salleh *et al*., 2002). *A. occidentale* has been used traditionally to treat various diseases including malaria, yellow

fever and diarrhea (Akinpelu, 2001; Concalves *et al*., 2005). Several phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, cinnamic, *p*-coumaric and ferulic acid were derived from *A. occidentale* leaves (Kogel and Zech, 1985). The anacardic acids that were previously isolated from *A. occidentale* have been shown to possess antibacterial, anticavity and antitumor properties (Green *et al*., 2008; Morais *et al*., 2010).

Antioxidant enzymes are capable of eliminating ROS and lipid peroxidation products by forming the first line defence, thereby protecting cells and tissues from oxidative damage. Antioxidant system in mammalian cells consists of three crucial enzymes namely, SOD, CAT and GPx that work in concert to detoxify ROS. The SOD converts superoxide radicals into hydrogen peroxide that is subsequently converted to water by CAT and GPx. In the present work, the activities of antioxidant enzymes increased in the cells due to H_2O_2 oxidation. These could be attributed to an instant role of these antioxidant enzymes in modulating the toxic effects of ROS. Previous studies showed that the antioxidant enzymes levels were reduced as overutilization to scavenge ROS (Manju and Nalini, 2005; Ihsan *et al*., 2011). However, pretreatment with AOE had reduced the antioxidant enzymes concentrations prior to the addition of H_2O_2 in HUVECs. Interestingly, the reduction of SOD, CAT and GPx activities in cells pretreated with AOE was found to be in dose dependent manner, with AOE at dose of 300 μg mL^{-1}, demonstrating the most effective antioxidant defence against lipid peroxidation induced by H_2O_2 on HUVECs. These findings suggest that AOE plays an active role in eliminating ROS, thereby reducing the need for enhanced antioxidant enzymes activities.

CONCLUSION

The results in this study showed that AOE possesses a free radical protective ability by reducing the cellular MDA production. The protective effect of AOE was postulated via enzymatic SOD, CAT and GPx activities in H_2O_2-induced HUVECs. These findings also suggested that AOE would act as a source of natural antioxidant that is potentially capable of attenuating early phase of free radical mediated diseases.

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