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Anacardium occidentale Aqueous Extract Attenuates Hydrogen Peroxide-Induced Oxidative Injury and Inhibits Inflammatory Mediators Expression in TNF-α-Induced Human Umbilical Vein Endothelial Cells During Initial Stage of Atherogenesis

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Abstract: Endothelial cell injury due to inflammation and oxidative stress are the hallmark of early pathologic events of atherosclerosis. Antioxidants derived from natural sources have been extensively used to prevent oxidative stress. The purpose of this study was to investigate the cytoprotective effect of *Anacardium occidentale* Aqueous Extract (AOE) against H_2O_2-induced HUVEC injury and its anti-inflammatory potential induced by TNF-α *in vitro*. HUVEC was 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% (IC_{50}) as denoted by MTT assay. Using the above concentration as the PC, the cells were pretreated with AOE at various concentration (50-700 μg mL^{-1}) for 30 min followed by 24 h incubation with H_2O_2 (250 μM) or TNF-α (10 ng mL^{-1}), respectively. AOE was found to be not toxic to the cells as no inhibitory concentration (IC_{50}) obtained. AOE (100-300 μg mL^{-1}) protects cellular damage and prevent microsomal lipid peroxidation in H_2O_2-induced HUVEC indicated with low MDA levels. The AOE at similar concentrations also suppressed the production of VCAM-1, ICAM-1, MCP-1 and M-CSF in TNF-α-induced inflammation whereas NF-κB p65 translocation into nucleus was observed inactivated. These data suggested that AOE possessed antioxidative properties and attenuate the initial stage of atherogenesis *in vitro*. Inhibition of NF-κB activation could be the possible underlying mechanism in modulating early events of atherogenesis.

Key words: *Anacardium occidentale*, HUVEC, antioxidant, anti-inflammation, atherogenesis, atherosclerosis

INTRODUCTION

Initial stage of atherogenesis is characterized by overexpression of cell adhesion molecules with subsequent accumulation of macrophages, smooth muscle cells and proliferation of extracellular matrix in the arterial intima. The process of atherogenesis is still not completely understood but it is generally believed that oxidative stress and inflammation derived from free radical attack to the biological system plays an important role in all stages of atherosclerosis. Reactive Oxygen Species (ROS) also play a central role in atherosclerosis and their effects on vascular function depend critically on the amounts produced.

Chronic inflammatory reaction in the arterial wall is usually accompanied by the recruitment of macrophages and lymphocytes in the arterial intima. This accumulation is strongly regulated by the expression of various cell adhesion molecules including VCAM-1 and ICAM-1. Cytokines such as interleukin-1 (IL-1) and TNF-α produced in acute and chronic conditions are important mediators of induced adhesion molecules expression. TNF-α stimulates NF-κB activation and induces the expression of inflammatory response genes including VCAM-1 and MCP-1 (Bergh *et al*., 2009; Pan and Dai, 2009). The activation of NF-κB causes localization of endothelial expression of VCAM-1 and ICAM-1 resulting with the adherence of monocytes to the developing lesion area (Kitagawa *et al*., 2002; Blankenberg *et al*., 2003). Subsequent conversion of monocytes to foamy macrophages results in the synthesis of a wide variety of inflammatory cytokines, growth factors and chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) that causes the formation of mature

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atherosclerotic plaques (Blasi, 2008). On the other hand, Macrophage Colony Stimulating Factor (M-CSF) influences hemopoietic stem cells to differentiate into macrophages or other related cell types. The plasma levels of M-CSF have been found elevated in patients with chronic arterial disease, unstable angina and acute myocardial infarction (Ikonomidis *et al*., 2008). *Anacardium occidentale* or gajus in Malay is a well known traditional medicine within Asean region and has been used traditionally to treat many diseases such as diabetes, diarrhea, malaria and yellow fever (Akinpelu, 2001; Concalves *et al*., 2005). The plant is an ornamental tree up to 10 m high. Phytochemical studies of *A. occidentale* have revealed the presence of various compounds such as flavonoids, glycosides and glucose (Konan and Bacchi, 2007) which possesses high antioxidant activities (Nurhanani *et al*., 2008). Even though, there were many antioxidant activities and phenolic content of this plant have been reported but the focus were mainly in the nuts and stem barks (Kornsteiner *et al*., 2006; Kubo *et al*., 2006). The leaves were commonly consumed but limited information on the antioxidant activities and its potential effect on atherosclerosis. In view of this, the aim of this study was to provide further information on the effect of *A. occidentale* Aqueous Extract (AOE) in the initial stage of atherosclerosis using HUVEC as a model *in vitro*. This study hypothesized that AOE could act as antioxidant and anti-inflammatory agent to prevent oxidative-derived cell injury and attenuate the early episode of atherogenesis.

MATERIALS AND METHODS

Preparation of 10% *A. occidentale* aqueous extract: The fresh leaves of *A. occidentale* were sourced from Forest Research Institute of Malaysia 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. Ten percent of *A. occidentale* aqueous extract was prepared by soaking 100 g of the powdered leaves in 1000 mL distilled water and incubated 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 (HUVEC) were purchased from American Type Culture Collection (ATCC, Rockvilled, MD, USA). The cells were maintained in a T-25 flasks (Nunc, Roskilde, Denmark) containing M200 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 as described by Chen *et al*. (2004) with slight modification. 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 oder to obtain the inhibitory concentration of H_2O_2 or toxicity potential of AOE on HUVEC, 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, 500, 600 and 700 μM is added and incubated for the subsequent 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 Sulphoxide (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:

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

Experimental protocol for oxidative stress and inflammation: At the initial time of the experiment, HUVEC (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 new culture medium 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) or TNF-α (10 ng mL^{-1}), respectively. Positive Control (PC) was denoted as cells treated with H_2O_2 or TNF-α alone without the extracts, whereas Negative Control (NC) was cells with only the culture medium. After the incubation period, the cells were homogenated; the cell lysate obtained was used for the determination of biochemistry assay.

Level of MDA: 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.

Expression levels of VCAM-1, ICAM-1, MCP-1 and M-CSF: The procedure was based on the method according to commercially available ELISA kit (BMS232, BMS201, Bender Med System, Austria and ELH-MCP1-001, Ray Biotech Inc, USA), respectively.

NF-κB activation: Briefly, the cells were disrupted in order to obtain the nuclear extract according to the kit protocol (2900, Chemicon International, USA). Then, the p65 NF-κB activation was determined colorimetrically using commercially kit following the instructions of the manufacturer (70-501, Upstate® Universal EZ-TFA Transcription Factor Assay Colorimetric, Millipore Inc, USA).

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

RESULTS AND DISCUSSION

The used of herbs or herbal-based product for the treatment of different diseases had been reported for hundreds of years. Although, many herbal medicines have been effective in the treatment of disease, often the mechanisms of action are largely unknown. The present investigation assessed a commonly prescribed preparation from AOE in oxidative stress injury and inflammation involved in the initial stage of atherogenesis by using HUVEC.

A. occidentale aqeous extract showed significantly reduced oxidative-derived cell injury and cell signalling molecules expression which involved in the initial events of atherogenesis *in vitro*. Exposure of H_2O_2 (250 μM) to HUVEC was observed to cause 50% cell death (Fig. 1) and that concentration was used as PC for the subsequent experiment. The IC_{50} of H_2O_2 that was determined from this experiment was near to the concentration of H_2O_2 obtained for the metabolic control analysis (Makino *et al*., 2004). However, researchers had found that exposure to 1 μM H_2O_2 to HUVEC for 20 min reduced cell viability to 55.8±1.9% of control (Stephen and Alan, 2003). Conversely, incubation of HUVEC with AOE at all concentration range for 24 h was showed to be not toxic as no cell death observed hence no IC_{50} value for AOE obtained (Fig. 2). Furthermore, it is important to mention that AOE used in the study has no effect on HUVEC, when the cells were exposed even to the highest

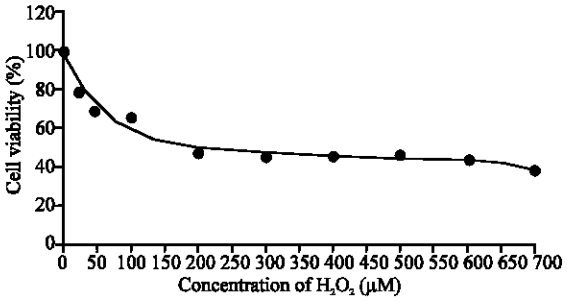

Fig. 1: Effect of H_2O_2 on HUVEC. The percentage of cell viability against concentration of H_2O_2 with different concentrations from 10-700 μg mL^{-1}

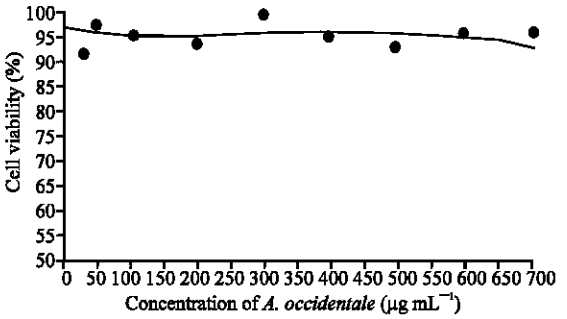

Fig. 2: Effect of AOE on HUVEC. The percentage of cell viability against concentrations of *A. occidentale* with different concentrations from 10-700 μg mL^{-1}

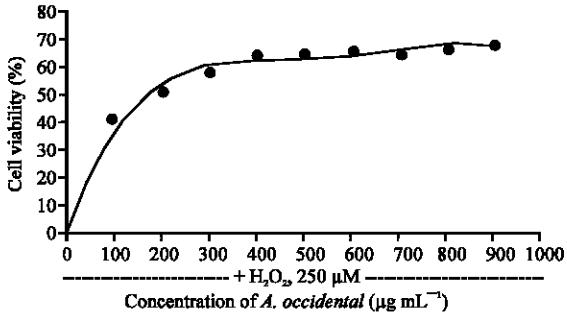

Fig. 3: Protective effect of AOE on H_2O_2-induced HUVEC oxidation. The percentage of cell viability treatment of AOE with different concentrations from 100-700 μg mL^{-1}

concentration (700 μg mL^{-1}) of AOE. This could highlight the safety use of AOE on HUVEC. In addition, cytoprotective effect of AO on H_2O_2-induced cell injury was found to be effective at 180 μg mL^{-1} (Fig. 3). The inhibitory effect of AOE against H_2O_2 induced cell oxidation was found to be in dose dependent manner. MDA is a by-product of lipid peroxidation induced by excessive ROS and widely used as a biomarker of oxidative stress. On the other hand, much evidences has

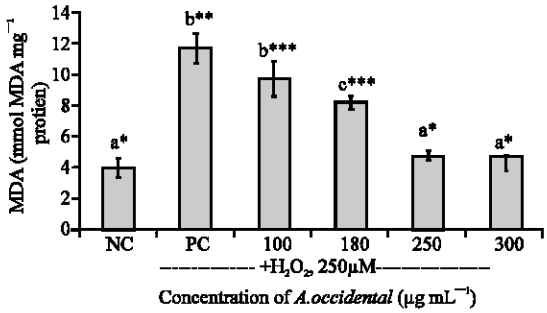

Fig. 4: Effect of AOE on level of MDA in H_2O2-induced HUVEC. Data were expressed as mean+SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

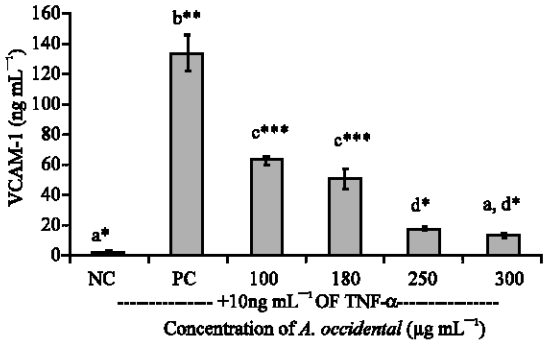

Fig. 5: Effect of AOE on adhesion molecule expression level of VCAM-1 in TNF-α-induced HUVEC. Data were expressed as mean±SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

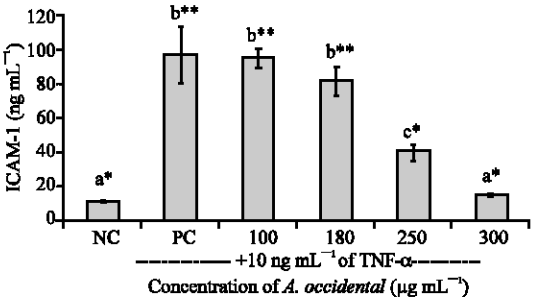

Fig. 6: Effect of AOE on adhesion molecule expression level of ICAM-1 in TNF-α-induced HUVEC. Data were expressed as mean±SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

revealed that H_2O_2 can cause endothelial cell injury by inducing mitochondrial dysfunction and cell death. H_2O_2 is described as non-free radical but its reactivity is very strong resembling ROS as it can generate hydroxyl radicals (Claudia *et al*., 2009). Pretreatment of AOE on HUVEC following exposure to 250 μM H_2O_2 decreased MDA level significantly as compared to cells without treatment (PC). As shown in Fig. 4, H_2O_2 clearly causes cellular injury and lipid peroxidation indicated with a significant increased of MDA concentration in three folds increment compared to the NC group ($p<0.05$ and 0.01). However, AOE demonstrated a protective effect against H_2O_2-induced oxidative stress and exhibit antioxidative properties with remarkable 3 folds reduction of MDA concentration in all treatment groups compared to the PC group ($p<0.05$ and 0.01). Previous studies reported that endothelial cells in human atherosclerotic lesions have been shown to increase Cell Adhesion Molecules (CAMs) expression such as ICAM-1 and VCAM-1. In normal control group, HUVEC produced VCAM-1 at very low level (Fig. 5). In PC group, the TNF-α (10 ng mL^{-1}) exposure caused significantly increased production of VCAM-1 (134.2±12.02 ng mL^{-1}). However, pretreatment with AOE significantly attenuated the TNF-α-induced VCAM-1 by about 2-7 fold less than PC group ($p<0.05$ and 0.01). In experiment of ICAM-1, PC group showed a significant increased on the production of ICAM-1 (96.16±16.3 ng mL^{-1}) compared to normal control group ($p<0.05$ and 0.01) (Fig. 6). However, all AOE pre-treatment groups demonstrated a significantly decreased of the TNF-α-induced ICAM-1 compared to PC group.

On the other hand, the recruitment of leukocytes to endothelial site is mediated by an increased gradient of chemotactic factors released from the endothelium by inflammatory stimuli. Exposure of HUVEC to TNF-α caused an up-regulation of MCP-1 compared to normal control group (Fig. 7). However, the effect of AOE was non-concentration dependent as the expression of MCP-1 was not significantly different to each other. This study demonstrated that the expression of MCP-1 in HUVEC after AOE treatment was reduced. On the other hand, the pretreatment of 100 μg mL^{-1} AOE was observed significantly decreased the M-CSF released compared to the PC group ($p<0.05$ and 0.01) (Fig. 8). These results indicated that AOE is an effective protector against monocyte recruitment in inflammatory vessel by suppressing both MCP-1 and M-CSF, which are likely to be as a potential therapeutic agent for atherosclerosis.

Previous studies have shown that TNF-α causes NF-κB activation (Madge and Pober, 2000; Tak and Firestein, 2001). In this study, TNF-α-induced NF-κB p65 translocation in HUVEC (PC) (Fig. 9). This finding was in

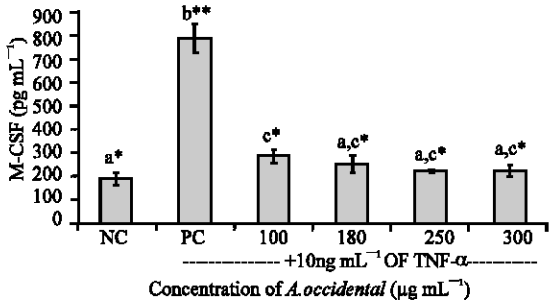

Fig. 7: Effect of AOE on expression level of M-CSF in TNF-α-induced HUVEC. Data were expressed as mean±SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

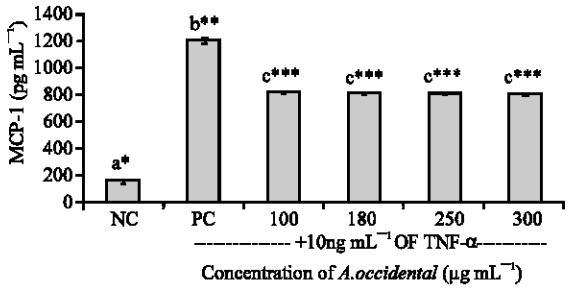

Fig. 8: Effect of AOE on expression level of MCP-1 in TNF-α-induced HUVEC. Data were expressed as mean±SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

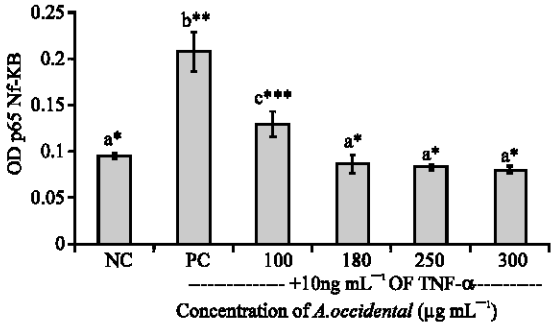

Fig. 9: Effect of AOE on activation NF-κB in TNF-α induced HUVEC. Data were expressed as mean±SD. Values with same letter were not significantly different between samples ($p<0.05$). Values with same (*) were not significantly different between samples ($p<0.01$)

accordance to previously reported data (Madge and Pober, 2000). However, pre-incubation of HUVEC with 100 μg mL^{-1} AOE for 30 min prior to TNF-α stimulation was significantly suppressed the NF-κB activation denoted with reduction of p65 nuclear translocation ($p<0.05$ and 0.01). In 180, 250 and 300 μg mL^{-1} AOE treatment groups, NF-κB activation was significantly different to PC and 100 μg mL^{-1} AOE treatment group ($p<0.05$ and 0.01).

The inhibitory effect was found to be dose-dependent manner. Previous studies reported that medicinal plant extract such as rhubarb suppressed NF-κB activation in vascular endothelial inflammation process (Moon *et al*., 2006). Hence, it was expected that AOE may possesses inhibitory effects on NF-κB activation and this could be the possible underlying mechanism of CAMs suppression.

In the study, a crude extract from AOE was used to treat HUVEC. Therefore, a limitation of this study is the inability to determine the specific components of the herb that mediate the effects observed. However, polyphenols and phenolics that rich in AOE would be the key player in reducing the oxidative stress derived from free radical attack to the biological system. As previous study has reported the presence of several phenolic acids in the leaves of AOE, mainly gallic acid, protocatechuic acid, p-hydroxybenzoic acid, cinnamic acid, p-coumaric acid and ferulic acid, which may act as antioxidant (Kogel and Zech, 1985).

CONCLUSION

The results in this study demonstrated that AOE might be an effective remedy to prevent oxidation of vascular endothelium cell and atherogenesis by inhibiting ROS production and overexpression of several cell adhesion molecules *in vitro*. Thus, AOE could be potentially useful in attenuating early events of atherosclerosis.

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