Protective effects of *Tinospora crispa* extracts on H$_2$O$_2$-induced oxidative stress and TNF-α-induced inflammation on human umbilical vein endothelial cells (HUVECs)

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Atherosclerosis is a pathology related to oxidative stress and inflammation. Prevention of atherosclerosis can be achieved by blocking the oxidation and inflammation process that occurs on the endothelial cells. *Tinospora crispa*, a climber that is commonly found in primary rainforest is widely distributed in Malaysia, Indonesia, Thailand and Vietnam. *T. crispa* which has been claimed to exert various health promoting effects has been reported to possess anti-diabetic, anti-malarial and anti-inflammatory properties. This study sought to investigate the effect of *T. crispa* extracts in preventing oxidative stress and inflammation induced by hydrogen peroxide (H$_2$O$_2$) and tumor necrosis factor (TNF)-α, respectively. Human umbilical vein endothelial cells (HUVECs) were cultured on 6-wells plate before treated by TCAE and TCME at various concentrations (100, 200, 400 and 600 µg/ml). After 30 min of incubation, H$_2$O$_2$ or TNF-α (10 ng/ml) was administered on HUVECs. HUVECs were harvested after 24 h and tested for CAT, SOD, GPx, MDA, ICAM-1, VCAM-1 and NO using various kit. The result showed that TCAE and TCME showed modulatory effect on H$_2$O$_2$-induced CAT, SOD and GPx activity. Concomitantly, MDA level was reduced by pretreatment of TCAE and TCME for 24 h. In addition, TCAE and TCME showed an inhibitory effect on TNF-α induced secretion of ICAM-1, VCAM-1 signaling molecule while NO secretion was increased. Therefore, these results showed a protective effect of *T. crispa* extracts on the H$_2$O$_2$-induced oxidative stress and TNF-α-induced inflammation.

Key words: Atherosclerosis, *Tinospora crispa*, oxidative stress, inflammation, H$_2$O$_2$, TNF-α, human umbilical vein endothelial cells (HUVECs).

INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality in Malaysia (Zambahari, 2004).

Cardiovascular disease such as coronary heart disease, stroke and myocardial infarction is the clinical expression of advanced atherosclerosis (Levy, 1981). Atherosclerosis is a pathological state related to oxidative stress (Shi et al., 2000; Upston et al., 2003) and inflammation (Reape and Groot, 1999) characterized by the focal development of atherosclerosis lesions in large
Tinospora crispa, locally known as patawali or andawali is a climber that can be found in primary rainforest widely distributed in Malaysia, Indonesia, Thailand and Vietnam. T. crispa has been traditionally used to treat diabetes, hypertension and lumbago by an ethnic group in Malaysia. T. crispa is previously reported to have antidiabetic (Noor and Ashcroft, 1998), antimalarial (Najib Nik A Rahman et al., 1999) and anti-inflammatory (Sulaiman et al., 2008) properties. Recent study by Praman et al. (2011) showed hypotensive effect of n-butanol extract from T. crispa.

This study sought to investigate the anti-atherosclerotic effect of Tinospora crispa aqueous (TCAE), and methanol extract (TCME) of T. crispa in particulars to its anti-oxidant and anti-inflammatory potentials. For that purposes, the effect of TCAE and TCME on expressions of endogenous antioxidant enzyme (CAT, SOD and GPx) as well as the release of malondialdehyde (MDA) stimulated with hydrogen peroxide (H₂O₂) in HUVECs were evaluated.

Furthermore, the level of adhesion molecule (ICAM-1 and VCAM-1) and nitric oxide (NO) production by TNF-α stimulated HUVECs were also studied.

MATERIALS AND METHODS

Endothelial cell medium (ECM) kit and trypsin-EDTA were purchased from ScienceCell (USA). Fetal bovine serum (FBS) was purchased from Gibco (USA).

Phosphate buffer saline (PBS), dimethyl sulphoxide (DMSO) and hydrogen peroxide (H₂O₂) were purchased from System (Malaysia). 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), butylated hydroxyl toluene (BHT), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and bovine serum albumin (BSA) was purchased from Sigma-Aldrich (USA), tumor necrosis factor-α (TNF-α) was purchased from Peprotech (USA). SOD assay kit was purchased from Sigma-alrich (USA). CAT, GPx and NO assay kits were purchased from Cayman chemicals (USA). ICAM-1 and VCAM-1 assay kits were purchased from Bender Medsystem, Austria. Von Willebrand Factor was purchased from Immunootech (FRA). BCA protein assay kit was purchase from Thermo Scientific (USA).

Preparation of T. crispa extract

T. crispa stems were collected from local forest from entire Malaysia. The stems were identified and authenticated by plant taxonomist from Forest Research Institute of Malaysia (FRIM). A voucher specimen was deposited in FRIM herbarium, KEP (FRI 54832).

The stems were washed thoroughly in tap water, cut into small pieces, air dried for 24 h and pulverized. Aqueous extract of T. crispa stems was prepared by soaking 100 g of the powdered T. crispa in 1000 ml distilled water and incubated in shaking water bath at 60°C for 6 h. Once filtered, the filtrate was freeze dried and kept at -20°C until used. Methanol extract of T. crispa stems was prepared by similar approach as the aqueous extract, with exception the incubation parameter was set at 25°C on an orbital shaker at 150 rpm for 24 h. The supernatant was filtered through filter paper. The residue was then extracted twice with additional...
1000 ml of methanol as described above. The combined methanol extracts were then subjected to rotary evaporated at 40°C to dryness and kept in the dark at 4°C until used.

**Cell cultures procedures**

Experimental HUVECs used in this study were purchased from Sciencell, USA. HUVECs were cultured by Endothelial Cell Medium (ECM) kit supplemented with 5% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, and 1% Endothelial Cell Growth Supplement (ECGS). HUVECs were grown to confluence at 5% CO₂ humidified incubator on a 75 cm² tissue culture flasks at 37°C at dark and were routinely subcultured in every 2-3 days as described by Chen et al. (2004). HUVECs were identified by their typical cobblestone morphology and immunofluorescence staining by monoclonal antibodies against Von Willebrand Factor (Marin et al., 2001). Cells up to the fourth passage were used for all experiments.

**MTT assay**

HUVECs were seeded at 1x10⁴ cells per well in 96 wellplates (Iwaki, Japan). After 24 h, the culture medium was replaced with ECM supplemented with 2% (v/v) FBS with or without TCAE and TCME at various concentrations (100, 200, 400 and 600 µg/ml). After that, the cultures were incubated for a further 24 h. HUVECs viability were determined according to the method of Mosmann (1983) with slight modification. Briefly, HUVECs were incubated with 0.5 mg/ml methyl thiazoltetrazolium salt (MTT) for 4 h at 37°C. The formazan crystals resulting from the MTT reduction were dissolved by adding 100% dimethyl sulphoxide (DMSO) and gentle agitation for 30 min. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 540 nm.

**Determination of the ability of *T. crispa* extracts to attenuate the cytotoxic effect of H₂O₂**

HUVECs were seeded at 1x10⁴ cells per well in 96 wellplates. After 24 h, the culture medium was replaced with ECM supplemented with 2% (v/v) FBS with or without TCAE and TCME at various concentrations (100, 200, 400 and 600 µg/ml). After 30 min, 250 µM of H₂O₂ were added into the wells. HUVECs were then incubated for another 24 h before proceeding to MTT assay. MTT assay was performed as described earlier.

**Experiment protocol for CAT, SOD, GPx and MDA.**

HUVECs were seeded at 1x10⁵ in 6 wellplate. After 24 h, the medium were replaced with ECM supplemented with 2% FBS with or without TCAE and TCME at various concentrations (100, 200, 400 and 600 µg/ml). After 30 min of pre-incubation of TCAE and TCME, 250 µM H₂O₂ was added to all well except for negative control well and HUVECs were further incubated for 24 h. Therefore, the group not incubated with H₂O₂ and extracts was denoted as negative control (NC) while the group incubated with H₂O₂ only was denoted as positive control (PC). After 24 h, cells from each well were washed by ice cold PBS, scrapped and transferred to falcon tube (Iwaki, Japan). Cells were then centrifuged at 1000 g for 10 min. The cells were then suspended with 1 ml of PBS and sonicated for 1 min. The cells can be used as a sample for further assay of antioxidant enzymes (CAT, SOD and GPx) and MDA assays.

**CAT, SOD and GPx analysis by enzyme-linked immunosorbent assay (ELISA)**

Concentration of CAT and GPx were quantified using kit purchased from Cayman, USA while SOD assay were quantified using kit purchased from Sigma-aldrich, USA. The assay was performed according to the instruction provided in kit manuals.

**MDA assay**

MDA assay was performed according to the method of Ohkawa et al. (1979) with slight modification. Briefly, 1 ml sample was added with 1.5 ml TBARS and 50 µl 2% BHT. TBARS reagent was prepared by adding 15% trichloroacetic acid (TCA) and 0.375% tibarbitruic acid (TBA) into 100 ml HCL 0.25 M. Then, the test tube was vortexed and boiled in water bath for 15 min at 100°C. Marble glass was placed on the tube opening to prevent tube mixture from exploding. After 15 min, the tube was cool down by running water. After that, 2 ml of n-butanol was added into each tube and vortexed for 3 min. Then, the test tube was centrifuged at 3000 rpm for 15 min. The upper layer (butanol) was taken and its absorbance was determined at 532 nm spectrophotometer. Values of MDA level were expressed as nanomoles per milligrams of protein. Protein concentration was measured with protein assay kit with BSA as standards.

**NO, ICAM-1, VCAM-1 analysis by enzyme-linked immunosorbent assay (ELISA)**

HUVECs were seeded and pretreated with various concentrations of samples as mentioned earlier. After 30 min of pretreatment of TCAE and TCME, 10 ng/ml TNF-α was added to all well except for NC group and HUVECs were further incubated for 24 h. After 24 h incubation periods, the cells supernatant were collected by centrifugation at 1000 rpm for 5 min. The cells supernatant were stored at -20°C for NO, ICAM-1 and VCAM-1 assays. Concentration of NO was quantified using commercially available kit purchased from Cayman, USA while concentration of ICAM-1 and VCAM-1 were quantified using kit purchased from Bender Medsystem, Austria. The assay was performed according to the instruction provided in kit manuals.

**Statistical analysis**

Statistical analysis was performed by one-way ANOVA with Turkey’s posthoc multiple group comparison using Statistical Package for Social Sciences software (SPSS 17, Chicago, IL, USA). P<0.05 were considered significant for all tests.

**RESULTS**

**Effects of TCAE and TCME on the viability of H₂O₂ treated HUVECs**

The effect of TCAE and TCME on the viability of H₂O₂ treated HUVECs were analyzed by MTT assay. Figure 1 shows that the survival rate of HUVECs was significantly decreased (about 50 %) after exposure to H₂O₂ (100 µM) for 24 h. However, pre-treatment of HUVECs with various concentrations of TCAE (50-1000 µg/ml) for 30 min has significantly improved the viability of HUVECs (up to
Figure 1. Protective effect of TCAE and TCME on H$_2$O$_2$-induced HUVECs. HUVECs were pre-incubated with 100-600 μg/ml TCAE or TCME for 30 min before exposure with 100 μM H$_2$O$_2$ and then incubated for 24 h. Values are mean ± SD (n=3). * are significantly different (P<0.05) compared to H$_2$O$_2$ treated cells.

Figure 2. Effect of TCAE and TCME on the A) CAT, B) SOD, and C) GPx enzyme activity in H$_2$O$_2$-induced HUVECs. HUVECs were pre-incubated with 100-600 μg/ml TCAE or TCME for 30 min before exposed with 100 μM H$_2$O$_2$ and then incubated for 24 h. Values are mean ± SD (n=3). * are significantly different (p<0.05) compared to H$_2$O$_2$ treated cells.

69%). Pretreatment with TCME has significantly increased the viability of HUVECs at the maximum of 76% at 600 μg/ml.

Level of antioxidant enzymes and MDA in H$_2$O$_2$-induced HUVECs

Exposure of HUVECs with H$_2$O$_2$ (100 μM) for 24 h markedly reduced the CAT, SOD and GPx activities. However, pre-treatment of TCAE (100, 200, 400 and 600 μg/ml) has modulated CAT, SOD and GPx activity in dose dependent manner (Figure 2a, b and c). Modulation of CAT and SOD in H$_2$O$_2$ treated HUVEC by TCME reached a maximum at 400 μg/ml. The increased GPx activity in H$_2$O$_2$ treated HUVEC by TCME in dose dependent manner, similar to TCAE action.

In addition, HUVECs exposed with 100 μM H$_2$O$_2$ for 24 h markedly increased the MDA level. Pre-treatment of HUVECs with TCAE (100, 200, 400 and 600 μg/ml) conversely attenuate the increased level of MDA with 15, 20, 43 and 58% of inhibition, respectively. TCME (100, 200, 400 and 600 μg/ml) showed a greater inhibition with 25, 34, 50 and 60% of inhibition, respectively.

Level of ICAM-1, VCAM-1 and NO in TNF-α-induced HUVECs

The effect of TCAE and TCME on the TNF-α-induced secretion of ICAM-1 and VCAM-1 on the surface of the HUVECs were evaluated using cell based ELISA.
Figure 3. Effect of TCAE and TCME on the MDA level in H$_2$O$_2$-induced HUVECs. HUVECs were pre-incubated with 100-600 μg/ml TCAE or TCME for 30 min before exposed with 100 μM H$_2$O$_2$ and then incubated for 24 h. Values are mean ± SD (n=3). * are significantly different (P<0.05) compared to H$_2$O$_2$ treated cells.

Figure 4. Effect of TCAE and TCME on the A) ICAM-1 and B) VCAM-1 level in H$_2$O$_2$-induced HUVECs. HUVECs were pre-incubated with 100-600 μg/ml TCAE or TCME for 30 min before exposed with 100 μM H$_2$O$_2$ and then incubated for 24 h. Values are mean ± SD (n=3). * are significantly different (P<0.05) compared to H$_2$O$_2$ treated cells.
Exposure of HUVECs with 10 ng/ml TNF-α for 24 h induced significant increased of ICAM-1 and VCAM-1 secretions. Pretreatment with TCAE markedly inhibits TNF-α induced secretion of ICAM-1 at 600 μg/ml (Figure) and inhibited TNF-α induced secretion of VCAM-1 at 100, 200, 400 and 600 μg/ml in-dose dependent manner. Pretreatment of TCAE at 400 and 600 μg/ml significantly inhibit TNF-α induced secretion of VCAM-1 while pretreatment with TME at 100, 200, 400 and 600 μg/ml significantly inhibit secretion of VCAM-1 in TNF-α induced HUVECs. Pretreatment of TCAE at 400 and 600 μg/ml significantly inhibit TNF-α induced secretion of VCAM-1 while pretreatment with TME at 100, 200, 400 and 600 μg/ml significantly inhibit secretion of VCAM-1 in TNF-α induced HUVECs.

To determine the ability of T. crispa extracts in promoting the release of NO in HUVECs, the concentration of NO in HUVECs treated with various concentration of TCAE and TME (100, 200, 400 and 600 μg/ml) was tested. Figure 5 shows that TCAE and TME caused significant increase of the NO level in dose-dependent manner compared to untreated groups.

DISCUSSION

Oxidative stress has been implicated in the pathogenesis of atherosclerosis. High level of ROS in blood circulation led to the oxidation of LDL and become the key event of atherosclerosis (Xu and Huang, 2007; Niki and Noguchi, 2002). H₂O₂, which is considered one of most powerful oxidant, was found to be involved in cellular signaling and regulation of HUVECs adhesiveness (Franzini et al., 1996). This oxidative insult on vascular tissue can be challenge by diet-derived antioxidant (tocopherol, carotenoids, ascorbate, flavonoids, Vitamin E) and plasma protein (Crosby et al., 1996; Praticò et al., 1998; Fennel et al., 2002; Hsieh et al., 2005). Endogenous antioxidant enzyme, particularly CAT, SOD and GPx play a key role in protecting cells and tissue from oxidative damage (Hertog et al., 1993; Venkatraman et al, 1994). In corroborate with that, this study sought to investigate, partly, the effect of TCAE and TME on H₂O₂-induced lipid peroxidation on HUVECs as well as on production of antioxidant enzymes such as CAT, SOD and GPx. Results of present study indicate protective effect of TCAE and TME against H₂O₂ induced toxicity in HUVECs. H₂O₂ is a precursor of other ROS and has been demonstrated by several studies to cause cell injury on HUVECs (Gong et al., 2010; Wang et al., 2010). In agreement with previous studies, this study showed decreased cell viability when HUVECs were exposed with H₂O₂. However, pretreatment with TCAE and TME have reduced toxicity effect of H₂O₂ and protect cells from injury. Furthermore, TCAE and TME showed modulatory effects on the H₂O₂-induced lipid peroxidation in HUVECs as evident from MDA evaluation. MDA, which has been widely used as a biomarker of oxidative stress.
is an indicator of lipid peroxidation and can be induced by excessive ROS (Luo and Xia, 2006). Measurement of MDA in this study was determined with TBARs reaction, in which it produced red pigment at pH 4.0 that can be estimated spectrophotometrically under wavelength 532 nm (Ohkawa et al., 1979). In this study, incubation of 100 μM H$_2$O$_2$ in HUVECs for 24 h increased MDA level compared to untreated HUVECs. Pretreatment of TCAE and TCME markedly reduce the MDA level in dose dependent manner. This result implied inhibitory properties of $T. crispa$ extract on lipid peroxidation induce by H$_2$O$_2$ on HUVECs.

With regard to antioxidant enzymes activities, HUVECs showed depleted CAT, SOD and GPx activities when exposed to H$_2$O$_2$ for 24 h. These results were in line with studies performed by other researchers which showed a reduction of antioxidant enzymes when HUVECs were stimulated with H$_2$O$_2$ (Lin et al., 2006; Liu et al., 2009; Chen et al., 2010). However, study by Erba et al., (2003) showed contrary result indicated with an increased level of antioxidant enzymes when HUVECs were exposed with another oxidative agent, Cu$^{2+}$. This variation can be explained by the compensatory mechanism of cells; a slight oxidative stress environment, whereas the activities decrease in severe oxidative stress environment (Erba et al., 2003). Intracellular antioxidant enzymes play a pivotal role in defense mechanism by cells in combating oxidative stress to the cellular damage by ROS, particularly H$_2$O$_2$ (Liu et al., 2009). In this study, pretreatment with TCAE and TCME showed a protective effect against H$_2$O$_2$-induced impaired antioxidant enzymes activities and found to be in agreement with previous studies (Niki and Noguchi, 2002; Lin et al., 2006; Liu et al., 2009; Chen et al., 2010). Taken together of this antioxidant activity and MDA results, this study suggest that $T. crispa$ extracts possesses vascular cells from oxidative damage by enhancing endogenous antioxidant enzymes and prevent lipid peroxidation. Nitric oxide (NO), a gas with a half-life of several seconds (Achan et al., 2003) is a potent endogenous vasodilator that derived from the metabolism of L-arginine (Ignarro et al., 1999). NO plays a role in mediating the regulation of the vascular tone and vascular homeostasis (Napoli and Ignarro, 2001). Under normal physiological conditions, a well-defined distribution of NO is maintained (Napoli and Ignarro, 2001). A reduction in NO synthesis or bioavailability may promote platelet adhesion and aggregation as well as deposition of platelets on the abnormal endothelial surface (Napoli and Ignarro, 2001), which will eventually contribute to the initiation and progressivity of atherosclerosis (Ignarro et al., 1999; Drexler, 1999; Cooke, 1998; Anderson et al., 1995). Similar to the result of current study by Gong et al. (2010), present study indicates $T. crispa$ extracts increase extracellular NO release in HUVECs compared to untreated groups suggesting a vascular vasodilatory effect $T. crispa$ thus preventing thus preventing the progression of atherosclerosis.

To further confirm the ability of $T. crispa$ extracts in preventing atherogenesis, this study investigated the effect of TCAE and TCME on the secretion of ICAM-1 and VCAM-1 in TNF-α-induced HUVECs. In agreement with previous study (Huang et al., 2007; Mo et al., 2007; Cao et al., 2009), exposure of TNF-α on HUVECs caused significant increase of ICAM-1 and VCAM-1 secretion by HUVECs. This support a well known theory that TNF-α, a proinflammatory cytokine, can induce or activate the endothelial cells to express adhesion molecules (Bevilacqua et al., 1989; Springer, 1990) and is commonly found in atherosclerotic lesions (DiDonato et al., 1999; Ross, 1999). ICAM-1 and VCAM-1 are two members of immunoglobulin supergene family (IgSF) that play a major role in the adhesion and migration of leukocytes upon activation by pro-inflammatory cytokine (Kampen and Mallard, 2001). Increased level of ICAM-1 and VCAM-1 has been implicated in early events of atherosclerosis (Price and Loscalzo, 1999; Blankenberg et al., 2003). Due to its remarkable role in inflammation, attenuation of ICAM-1 and VCAM-1 responsiveness to vascular cells remains a key anti-inflammatory target for atherosclerotic disease (Hu et al., 2000). In this study, pretreatment of TCAE and TCME have caused the suppression on the level of ICAM-1 and VCAM-1 expression in TNF-α-induced HUVECs. This result showed a beneficial effect of $T. crispa$ extracts in reducing ICAM-1 and VCAM-1 expression in endothelial cells thus the risk of atherosclerosis disease.

Based on traditional claims, $T. crispa$ possess many health promoting properties including prevention of diabetes, hypertension and lumbago diseases. Several researchers also have postulated the antidiabetic (Noor and Ashcroft, 1998), antimalarial (Najib Nik A Rahman et al., 1999) and anti-inflammatory (Sulaiman et al., 2008) activities of $T. crispa$ extracts. A study by Ibrahim et al. (2011) showed high DPPH radical scavenging activity of $T. crispa$ extracts which were attributed by their flavonoids and phenolics content. Praman et al. (2011) demonstrated hypotensive effect of n-butanol extract from $T. crispa$. However, to the best of our knowledge, no study on the effect of aqueous and methanol extracts from $T. crispa$ has been carried out ROS-induced endogenous antioxidant activites as well as on the secretion of ICAM-1 and VCAM-1 inflammatory markers stimulated by TNF-α. Collectively, results of this study showed protective effect of $T. crispa$ extracts from lipid peroxidation and secretion of adhesion molecules, as well as maintaining the level of antioxidant enzymes and NO synthesis by HUVECs. Therefore, this study serves as a preliminary data to further investigation on, the anti-atherosclerotic effect of $T. crispa$ extracts to the level of mRNA and protein expressions as well as its possible mechanisms of action.
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REFERENCES


