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Inhibitory Properties of *Tinospora crispa* Extracts on TNF-α Induced Inflammation on Human Umbilical Vein Endothelial Cells (HUVECS)

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Abstract: There were accumulating evidences that relate the occurrence of atherosclerosis and inflammation in the intima of arteries. Atherosclerosis itself has been known as chronic inflammatory disease. Many signaling molecules such as ICAM-1, VCAM-1, MCP-1, M-CSF and NO have been found in the atherosclerotic plague of the arteries in heart disease patients. This study aimed to investigate the effect of *Tinospora crispa* Aqueous Extract (TCAE) and Methanol Extract (TCME) on Tumor Necrosis Factor (TNF)-α induced inflammation on Human Umbillical Vein Endothelial Cells (HUVECs) *in vitro*. HUVECs were cultured on 6 wells plate before been treated by TCAE and TCME at various concentrations (100, 200, 400 and 600 μg mL^{-1}). After 1 h of incubation, TNF-α (10 ng mL^{-1}) was exposed on HUVECs. HUVECs were harvested after 24 h and tested for ICAM-1, VCAM-1, M-CSF and NO using kit. Results of this study indicated that TCAE and TCME exert inhibitory effect on TNF-α induced secretion of ICAM-1, VCAM-1 and M-CSF signaling molecule while NO secretion was increased. These results showed that *T. crispa* extracts has inhibitory effect *in vitro* on the level of inflammatory signaling molecules thus it may have a potential benefits on the development of nutraceuticals in the prevention of atherosclerosis-related cardiovascular diseases.

Key words: *Tinospora crispa*, TNF-α inflammation, HUVECs, cardiovascular diseases, Malaysia

INTRODUCTION

Atherosclerosis is a disease associated with inflammation (Reape and Groot, 1999). Increasing evidences support an involvement of inflammation in pathogenesis of atherogenesis (Libby *et al*., 2002). Adhesion molecule such as Intercellular Cell Adhesion Molecule (ICAM-1) and Vascular Cell Adhesion Molecule (VCAM-1) as well as inflammatory marker such as Macrophage Colony Stimulating Factor (M-CSF) were upregulated during immune response and immune surveillance (Kampen and Mallard, 2001). Those signaling molecules can be stimulated by pro-inflammatory cytokine like TNF-α (Kleinbongard *et al*., 2010).

Expressions of ICAM-1 and VCAM-1 on endothelium play a role in the recruitment of leukocyte to the endothelial cell (Springer, 1994; Proost *et al*., 1996). In humans, focal expression of adhesion molecules has been consistently observed in atheroslerotic plaques (Davies *et al*., 1993; O'Brien *et al*., 1993). The expression of adhesion molecules for the recruitment of leukocytes to the site of injury become a major event in the development of the pro-inflammatory state. Suppression of the pro-infammatory endothelial cell state will limit the atherosclerotic process (Zhang *et al*., 2002). M-CSF plays a pivotal role in the regulation of the proliferation, differentiation and survival of homopoietic progenitor into mature macrophage (Rettenmier and Sherr, 1989). M-CSF has been reported to influence various functions of mature mononuclear phagocytes (Kurland *et al*., 1979; Moore *et al*., 1980; Fleit and Rabinovitch, 1981) and is also a potent chemoattractant.

Nitric Oxide (NO), a gas with a half-life of several seconds (Achan *et al*., 2003) plays a role in mediating the regulation of the vascular tone. Under normal physiological conditions, a well-defined distribution of NO is maintained (Napoli and Ignarro, 2001). A reduction in NO synthesis and/or activity may contribute to the

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initiation and progressivity of atherosclerosis (Anderson *et al*., 1995; Cooke, 1998; Dexler, 1999; Ignarro *et al*., 1999).

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 (Rahman *et al*., 1999), antibacterial (Zakaria *et al*., 2006), antifilarial, antipyretic (Kongkathip *et al*., 2002), antihyperglycaemic, antinociceptive and anti-inflammatory (Sulaiman *et al*., 2008) activity. Present study by Praman *et al*. (2011) showed hypotensive effect of n-butanol extract from *T. crispa*. Therefore, current study aims to evaluate the ability of *Tinospora crispa* exracts to attenuate the TNF-α induced secretion of these adhesion molecule and inflammatory signaling molecule in HUVECs.

MATERIALS AND METHODS

Preparation of plant crude 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 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, dried in an open air for 24 h and pulverized. Aqueous extract of *T. crispa* stem 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 sdried and kept at -20°C until used.

Methanol extract of *T. crispa* stem 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 that were used in this study were purchased from ScienCell, USA. HUVECs were cultured by Endothelial Cell Medium (ECM) kit (ScienCell, USA) supplemented with 5% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin and 1% Endothelial Cell Growth Supplement (ECGS). The HUVECs were grown to confluence at 5% CO_2 humidified incubator on 75 cm^2 tissue culture flasks at 37°C at dark. HUVECs 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 (immunotech) (Marin *et al*., 2001). Cells up to the fourth passage were used for all experiments.

Measurement of ICAM-1, VCAM-1, M-CSF and NO: HUVECs were seeded at 1×10^5 cells/well in 6 well plate. After 24 h, the medium were replaced with ECM supplemented with 2% FBS with TCAE and TCME at various concentrations (100, 200, 400 and 600 μg mL^{-1}) except Positive Control (PC) and Negative Control (NC) wells. After 30 min of pre-incubation of TCAE and TCME, 10 ng mL^{-1} TNF-α was added to all wells except NC group and HUVECs were further incubated for 24 h. Therefore, the group contain HUVECs alone was denoted as NC while the group that contain HUVECs and TNF-α without extracts was denoted as PC. After 24 h incubation periods, the cells supernatant were collected and centrifuged at 1000 rpm for 5 min. The cells supernatant were stored at -20°C and can be used for ICAM-1, VCAM-1, M-CSF and NO assays.

Concentration of ICAM-1, VCAM-1 and M-CSF were quantified using commercially available kit purchased from Bender Medsystem, Austria while concentration of NO was quantified using kit purchased from Cayman, USA. 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). The $p<0.05$ and $p<0.01$ were considered significant for all tests.

RESULTS AND DISCUSSION

Level of ICAM-1 and VCAM-1 secretion 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. Exposure of HUVECs with 10 ng mL^{-1} TNF-α for 24 h induced significant increased of ICAM-1 and VCAM-1 secretions. Pretreatment with TCAE markedly inhibited ($p<0.05$) TNF-α induced secretion of ICAM-1 at 600 μg mL^{-1} (Fig. 1) and inhibited TNF-α induced secretion of VCAM-1 at 100, 200, 400 and 600 μg mL^{-1} in dose dependent manner. Pretreatment of TCAE at 400 and 600 μg mL^{-1} significantly inhibit TNF-α induced secretion

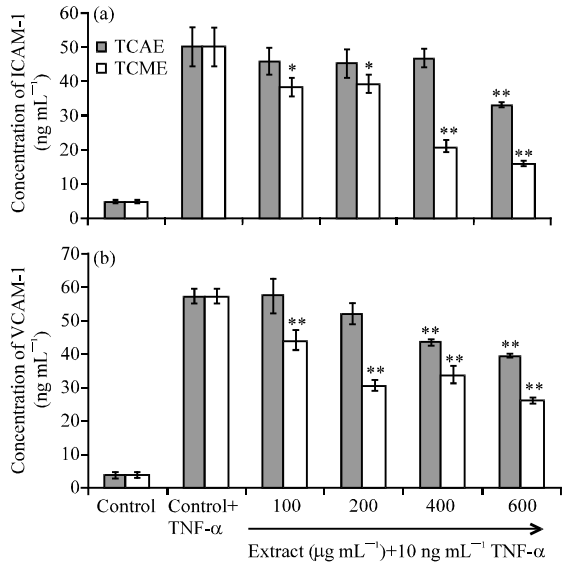

Fig. 1: Inhibition of; a) ICAM-1 and b) VCAM-1 secretion by TCAE and TCME in HUVECs. HUVECs were supplemented with different concentrations of TCAE and TCME. After 1 h incubation, cells were stimulated with/without TNF-α (10 ng mL^{-1}) for another 24 h. Culture supernatants were analyzed by ELISA as described. Data were expressed as the mean±SD Significantly different vs. TNF-α treated alone *p<0.05. Significantly different against TNF-α treated alone **p<0.01

of VCAM-1 while pretreatment of TCME at 100, 200, 400 and 600 μg mL^{-1} significantly inhibit secretion of VCAM-1 in TNF-α induced HUVECs.

Level of M-CSF secretion in TNF-α induced HUVECs: The expressions of M-CSF were induced 24 h after the TNF-α stimulation. TNF-α stimulated M-CSF secretion was inhibited when cells were pretreated with TCAE and TCME. Pretreatment of TCAE at 200-600 μg mL^{-1} significantly reduced ($p<0.05$) VCAM-1 release while pretreatment of TCME at 100-600 μg mL^{-1} significantly reduced ($p<0.01$) VCAM-1 release in dose-dependent manner (Fig. 2).

Level of NO secretion in TNF-α induced HUVECs: To determine the ability of *T. crispa* extracts in promoting the release of NO in HUVECs, the co concentration of NO in HUVECs treated with various concentration of TCAE and TCME (100, 200, 400 and 600 μg mL^{-1}) was tested. As shown in the Fig. 3, TCAE and TCME caused significant increase of the NO level in dose-dependent manner compared to untreated HUVECs.

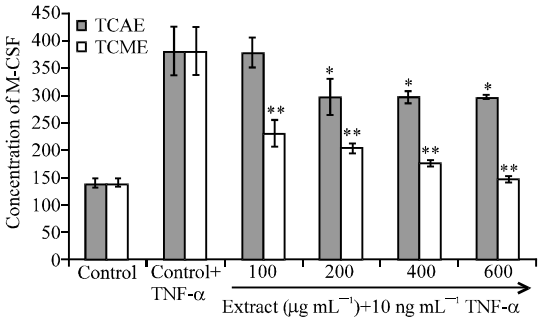

Fig. 2: Inhibition of M-CSF secretion by TCAE and TCME in HUVECs. HUVECs were supplemented with different concentrations of TCAE and TCME. After 1 h incubation, cells were stimulated with/without TNF-α (10 ng mL^{-1}) for another 24 h. Culture supernatants were analyzed by ELISA as described. Data were expressed as the mean±SD. Significantly different against TNF-α treated alone *p<0.05. Significantly different against TNF-α treated alone **p<0.01

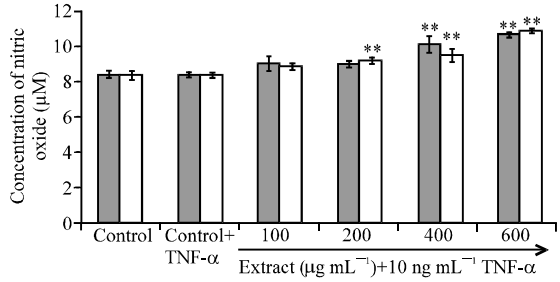

Fig. 3: Enhancement of NO secretion by TCAE and TCME in HUVECs. HUVECs were supplemented with different concentrations of TCAE and TCME. After 1 h incubation, cells were stimulated with/without TNF-α (10 ng mL^{-1}) for another 24 h. Culture supernatants were analyzed by ELISA as described. Data were expressed as the mean±SD. Significantly different against TNF-α treated alone *p<0.05. Significantly different against TNF-α treated alone **p<0.01

Previous study by the group has postulated anti-oxidative properties of *T. crispa* extracts which protect HUVECs from oxidative stress by means of CAT, SOD, GPx and MDA assay (Ihsan *et al*., 2011). That study supports the previous study which proposed the antioxidative properties of *T. crispa* extracts (Ibrahim *et al*., 2011). Since, atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress on the vascular endothelial cell, it is reasonable to conclude that the anti-atherosclerotic effects of *T. crispa* are due to its ability to support anti-oxidative defense

mechanisms. Present study analyzes the effect of TCAE and TCME on the expression level of adhesion molecule and inflammatory marker which was stimulated by pro-inflammatory cytokine, TNF-α. Stimulation of HUVECs by TNF-α increased the level of ICAM-1 and VCAM-1 expressions. This result was in accordance with the study performed by Mo *et al.* (2007) that showed TNF-α has induced the expression of ICAM-1 and VCAM-1 in HUVECs. Pro-inflammatory signals including cytokines and oxidative stress, along with several well established risk factors such as hyperglycemia, hyperlipidemia, hypertension and smoking, play prominent roles in the pathogenesis of coronary artery disease (Kunsch *et al.*, 2004).

The amount of ICAM-1 and VCAM-1 has been demonstrated to be directly correlated with the surface expression of ICAM-1 and VCAM-1 in endothelial cells in culture (Leeuwenberg *et al.*, 1992). Interactions of these molecules with their counterpart ligands on leukocytes mediate adhesion and extravasation at the site of inflammation (Vestweber and Blanks, 1999). Studies in humans and experimental animals have found that an increased expression of VCAM-1 and ICAM-1 is associated with an increased intimal leukocyte accumulation and that there is an abundance of adhesion molecules on arterial sites prone to the development of atherosclerotic lesions (Davies *et al.*, 1993; Johnson-Tidey *et al.*, 1994; Blankenberg *et al.*, 2003; DeGraba *et al.*, 1998; Takahashi *et al.*, 2002; Cybulsky *et al.*, 2004; Quehenberger, 2005). Results in this study showed the levels of ICAM-1 and VCAM-1 expressions in TNF-α induced HUVECs were suppressed by the supplementation of TCAE and TCME. These results indicate the ability of *T. crispa* extracts to attenuate the effect of TNF-α in promoting the expression of adhesion molecules thus prevent or delay the occurrence of atherosclerosis.

The study M-CSF expression showed TCAE and TCME suppresses the M-CSF expression in HUVECs in TNF-α induced HUVECs. M-CSF which regulates the proliferation, differentiation and survival of homopoietic progenitor into mature macrophage (Rettenmier and Sherr, 1989) plays a very important role in atherosclerosis. M-CSF can activate many functions of mature macrophages including accelerating extra-cellular cholesterol transportation (Yamada *et al.*, 1992), activating intra-cellular cholesterol hydrolyase and cholesterol ester transportase, regulating scavenger receptors at levels of gene expression, protein synthesis and protein stability (Parker *et al.*, 1990). Like ICAM-1 and VCAM-1, M-CSF is also inducible by TNF-α (Gruber and Gerrard, 1992). In present study, TCAE and TCME showed the ability to suppress the M-CSF level in TNF-α induced HUVECs. This data indicates that *T. crispa* extracts might reduce the risk of atherosclerosis-related diseases by preventing the survival and differentiation of monocyte into macrophage thus prevents the formation of foam cells.

NO, a potent endogenous vasodilator plays a pivotal role in vascular homeostasis (Ignarro *et al.*, 1999). Endothelium-derived nitric oxide induces vasodilation and which opposes inflammation, thrombosis and cellular proliferation. The loss of normal endothelium-dependent vasodilation results in abnormal vasomotion (Cooke *et al.*, 2000). Decreased NO bioavailability may disrupts the non-thrombogenic intimal surface and promotes platelet adhesion and aggregation as well as deposition of platelets on the abnormal endothelial surface (Napoli and Ignarro, 2001). This will contribute to the initiation and progressivity of atherosclerosis (Anderson *et al.*, 1995; Dexler, 1999; Ignarro *et al.*, 1999; Cooke *et al.*, 2000). Present study showed TCAE and TCME raise the NO levels in HUVECs compared to untreated groups. This result indicates *T. crispa* extracts could promote vascular vasodilatory effect thus preventing from the development of atherosclerotic plague.

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 (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 to their flavonoids and phenolics content. Praman *et al.* (2011) demonstrated hypotensive effect of n-butanol extract from *T. crispa*. However, to the best of researchers's knowledge this study was the first that investigates the effect of aqueous and methanol extracts on the on the secretion of ICAM-1,VCAM-1, M-CSF inflammatory markers stimulated by TNF-α as well as production of NO in HUVECs.

CONCLUSION

The results of this study showed protective effect of *T. cispa* extracts from 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 investigate in the future study, 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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