Research Journal of Biological Sciences 6 (5): 230-236, 2011
ISSN: 1815-8846
© Medwell Journals, 2011

# Antioxidant Properties of Aqueous and Methanol Extract from *Tinospora crispa* and its Ability to Attenuate Hydrogen Peroxide-induced Stress Injury in Human Umbilical Vein Endothelial Cells

[1]S.K. Ihsan, [2]H.A. Zulkhairi, [2]M.A. Abdah, [2]J.A. Daryl, [2]I. Noramalina, [1]M.A. Rasadah, [1]M. Shahidan, [1]M.S. Zamree, [1]K.A.K. Kamilah, [1]N.H. Mohd Kamal, [3]A. Azrina, [3]M.E. Norhaizan, [2]M.A.M. Aris and [2]M.B. Taufik
[1]Natural Product Division, Forest Research Institute of Malaysia, 52109 Kepong, Kuala Lumpur, Malaysia
[2]Department of Human Anatomy Division of Physiology, [3]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 U.P.M. Serdang, Selangor Darul Ehsan, Malaysia

**Abstract:** The aimed of this study was to investigate the antioxidant properties of *T. crispa* as well as its ability to attenuate the release of oxidant markers in induced oxidation in Human Umbilical Vein Cells (HUVECs). *In vitro* studies of antioxidant properties of *T. crispa* showed the DPPH scavenging activity of *T. crispa* Aqueous (TCAE) and Methanol Extract (TCME) were 82±1.78 and 73±1.01%, respectively. FRAP value of TCAE and TCME were 1.04±0.27 and 1.64±0.06 mmol $L^{-1}$, respectively. Total Flavonoids Content (TFC) of TCAE and TCME were 205.58±3.5 and 223±10.49 mg QE $g^{-1}$ sample, respectively while Total Phenolics Content (TPC) of TCAE and TCME were 32.58±0.68 and 41.64±0.97 mg GAE $mg^{-1}$ sample, respectively. The antioxidant enzymes activities (CAT, SOD and GPx) in HUVECs treated with *T. crispa* extracts to counter the oxidative effect by hydrogen peroxide ($H_2O_2$) and lipid peroxidation activity by Malondialdehyde (MDA) were also measured. The result showed TCAE and TCME at a concentration ranges from 100-600 μg $mL^{-1}$ significantly increased ($p<0.05$, 0.01) the level of CAT, SOD and GPx. Results of MDA assay showed significant reduction ($p<0.05$, 0.01) of MDA level in HUVECs treated with TCAE and TCME. Taken together, the findings suggest that *T. crispa* extracts can effectively protect HUVECs against oxidative stress by $H_2O_2$ which might be of importance in the treatment of cardiovascular diseases.

**Key words:** *Tinospora crispa*, HUVECs, anti-oxidant, anti-inflammatory, cardiovascular diseases, Malysia

## INTRODUCTION

Atherosclerosis is known to be associated with oxidative stress (Shi *et al*., 2006) and became a cause of death of approximately 16.7 million people (WHO, 2003). Atherosclerosis is the most prevalent disease responsible for death from myocardial infarction, cerebrovascular events, renal failure and ruptured aortic aneurysms (Glass and Witztum, 2001).

Imbalance between antioxidant and ROS in favour to the latter will cause oxidative stress. Oxidative stress alters diverse functional responses of endothelial cells and thereby is regarded as a critical pathogenic factor in the development of CVD (Malek and Izumo, 1994). There is increasing interest in the use of antioxidant agents against ROS stimulus in CVD which has been proved in studies of sphingosine 1-phosphate, propofol, Vitamin C (Bowie and O'Neill, 2000; Luo and Xia, 2006; Moriue *et al*., 2008). The endogenous enzymes such as Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) may also play a pivotal role in preventing cell injury cause by ROS (Luo and Xia, 2006). These antioxidant enzymes were inducible in a response to oxidative stress in various organisms and respond to oxidative stress by increase their antioxidant enzyme activities (Kotze, 2003). SOD and catalase work in tandem. SOD scavenges superoxide anion by forming hydrogen peroxide while CAT and GPx safely decompose hydrogen peroxide to water and superoxide anion.

This study aimed to determine the antioxidant potential of *T. crispa* in response to ROS activity *in vitro*. The 1, 1-Diphenyl-2-Picylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP) Assays Total Phenolics Content (TPC) and Total Flavonoids Content

**Corresponding Author:** S.K. Ihsan, Natural Product Division, Forest Research Institute of Malaysia, 52109 Kepong, Kuala Lumpur, Malaysia

(TFC) of *T. crispa* extract were carried out to provide pleminary data on its antioxidant properties while the antioxidant enzymes activity of the extract was assessed by evaluating the activity of CAT, SOD and GPx on human umbillical endothelial cells (HUVECs). MDA assay was also performed to determine the ability of *T. crispa* extracts to inhibit lipid peroxidation caused by ROS *in vitro*.

## MATERIALS AND METHODS

**Plant crude extract:** *T. crispa* plant was collected from local forest from entire Malaysia. The plant was identified and authenticated by plant taxonomist from Forest Research Instituite of Malaysia (FRIM). A voucher specimen was deposited in FRIM herbarium, KEP (FRI 54832).

The stems of *T. crispa* 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 dried 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 temperature was set at 25°C on an orbital shaker at 150 rpm for 24 h. The supernatant was filtered using 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 dryness using rotary evaporator at 40°C to and kept in the dark at 4°C until used.

**1,1-Diphenyl-2-Picylhydrazyl (DPPH) assay:** Determination of antioxidant activity by DPPH radical scavenging method was carried out using a method described by Yen and Hsieh (1998). Briefly, 0.45 mM DPPH was prepared by dissolving in absolute ethanol. About 1 mL of the 0.45 mM DPPH was added into 0.5 of each sample. Butylated Hydroxyl Toluene (BHT) and vitamic C were used as positive control and absolute ethanol was used as negative control. All the samples were prepared in triplicates. The decrease of absorbance was monitored after 30 min incubation period at the wavelength 517 nm at room temperature. The antioxidant activity was expressed using following equation:

$$\text{Scavenging effect (\%)} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right)$$

**Ferric Reducing Antioxidant Potential (FRAP) assay:** The FRAP assay was performed according to the method of Benzie and Strain (1996). It depends on the ability of the sample to reduce the ferric tripyridlytriazine (Fe (III)-TPTZ) complex to ferrous tripyridyltriazine (Fe(II)-TPTZ) at low pH. Fe(II)-TPTZ has an intensive blue color which can be read at 593.3 mL FRAP reagent was taken and the absorbance was read at 593 nm and set as the blank. Then, 100 μL of samples were added to FRAP reagent with the addition of 300 μL of distilled water. The mixture was left for 4 min and the absorbance was read at 593 nm. Aqueous solution of known Fe(II) concentration was used for calibration. A standard curve was plotted at 500, 1000, 2000 and 2500 $\mu$mol $L^{-1}$.

**Determination of Total Phenolics Content (TPC):** The determination of total phenolic content was carried out using this Folin-Ciocalteu method as described by Velioglu *et al.* (1998). Briefly, 200 μL of the extract with concentration of 0.2 mg $mL^{-1}$ was dissolved in 0.75 mL of 6% sodium carbonate ($Na_2CO_3$) solution. The final mixture was shaken and then incubated for 90 min in a dark room at room temperature. The absorbance of all samples was measured at 725 nm using UV-Vis spectrophotometer. Gallic acid was used as a reference. Standard calibration curve was plotted at 0.02, 0.04, 0.06, 0.08 and 0.10 mg $mL^{-1}$ gallic acid that was prepared in 80% (v/v) methanol. The absorbance was recorded at 725 nm using 80% (v/v) methanol as a blank. Total phenolic content was expressed as milligram of Gallic Acid Equivalents (GAE) per 100 g of samples.

**Determination of Total Flavonoids Content (TFC):** The total flavonoid content was determined using the Dowd method as described by Zhishen *et al.* (1999) with slight modification. Briefly, 5 mL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of a *T. crispa* extracts solution (1 mg $mL^{-1}$). Absorption readings at 415 nm (Shidmadzu, Japan) were taken after 10 min against a blank sample consisting of a 5 mL honey solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with quercetin (Sigma-aldrich, St. Louis, USA) (0-50 mg $L^{-1}$) as the standard. The mean of three readings was used and expressed as mg of Quercetin Equivalents (QE)/g of extracts.

**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 and 37°C at dark. HUVECs were routinely subcultured in every 2-3 days as described by Chen. HUVECs were identified by their typical cobblestone morphology and immunofluorescence staining by monoclonal antibodies against Von Willebrand Factor (Immunotech). Cells up to the fourth passage were used for all experiments.

**MTT assay:** HUVECs were seeded at $1\times10^4$ cells per well in 96 wellplates (Iwaki, Japan). After 24 h, the culture medium was replaced with ECM supplemented with 2% (v/v) FBS without or with *T. crispa* Aqueous Extract (TCAE) and *T. crispa* Methanol Extract (TCME) at the concentrations of 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 $mL^{-1}$ 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 (VersaMax™).

**Determination of the ability of *T. crispa* extracts to attenuate the cytotoxic effect of $H_2O_2$:** HUVECs were seeded at $1\times10^4$ cells per well in 96 wellplates (Iwaki, Japan). After 24 h, the culture medium was replaced with ECM supplemented with 2% (v/v) FBS without or with *T. crispa* Aqueous Extract (TCAE) and *T. crispa* Methanol Extract (TCME) at the concentrations of 100, 200, 400 and 600 μg $mL^{-1}$. After 30 min, 250 μM of $H_2O_2$ were added into the wells. HUVECs were then incubated for another 24 h before proceed to MTT assay.

**Experiment protocol for oxidative stress:** HUVECs were seeded at $1\times10^5$ in 6 well plate. After 24 h, the medium were replaced with ECM supplemented with 2% FBS without or with TCAE and TCME at the final concentration of 100, 200, 400 and 600 μg $mL^{-1}$. The wells that have no extracts were used as negative control. After 30 min of pre-incubation of TCAE and TCME, 250 ug $mL^{-1}$ $H_2O_2$ was added to all well except for negative control well and HUVECs were further incubated for 24 h. 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 enzyme and Malondialdehyde Assay (MDA).

**CAT, SOD and GPx assay:** The procedure of CAT, SOD and GPx assay was based on the method according to commercially available Catalase Assay kit (Cayman, 707002, USA), SOD Determination kit (Sigma-aldrich, 19160, Switzerland) and Glutathione Peroxidase Assay kit (Cayman, 703102), respectively.

**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 into the waterbath for 15 min at temperature of 100°C. Marble glass was placed on the tube opening to prevent tube mixture from exploded out. 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 distance of 532 nm using spectrophotometer.

**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 16, Chicago, IL, USA). $p<0.05$ were considered significant for all tests.

## RESULTS AND DISCUSSION

Previous epidemiological studies documented on the effect of antioxidant in the prevention of atherosclerosis-related diseases (Hsieh *et al.*, 2005; Fennell *et al.*, 2002; Pratico *et al.*, 1998). Those studies also suggest that consumption of fruit and vegetable-rich diet inversely correlates with the risk of cardiovascular diseases. These chemoprotective effects are at least in part, related to the antioxidant activities of polyphenolic compounds, carotenoids or vitamins E and C (Hollman and Katan, 1999; Prior and Cao, 2000). *T. crispa*, locally known as Patawali in Malaysia has been traditionally used to treat diabetes, hypertension and lumbago.

*T. crispa* is reported to have anti-diabetic (Noor and Ashcroft, 1998), anti-malarial (Rahman *et al.*, 1999) and anti-bacterial activity (Zakaria *et al.*, 2006). Sulaiman *et al.* (2008) demonstrated anti-inflammatory of *T. crispa* in

Table 1: *In vitro* antioxidant activity, phenolics content and flavonoids content of TCAE, TCME, vitamin C and BHT

| Values | TCAE | TCME | Vitamin C | BHT |
|---|---|---|---|---|
| DPPH (%) | 81.65±1.78[a] | 73.21±1.010[b] | 92.02±0.41[c] | 91.41±0.25[c] |
| FRAP (mmol $L^{-1}$) | 1.04±0.27[a] | 1.64±0.060[a] | 3.87±0.47[c] | - |
| Total polyphenols (mg GAE $mg^{-1}$ sample) | 32.58±0.68[a] | 41.64±0.970[b] | - | - |
| Total flavonoids (mg QE $g^{-1}$ sample) | 205.58±3.50[a] | 223.00±10.49[a] | - | - |

Each value represents the mean±SD. Values with the same alphabet are not significantly different ($p<0.05$) between assays. GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent

various animal models. Moreover, recent study by Praman *et al.* (2011) reported hypotensive activity of *T. crispa*. Therefore, *T. crispa* was found to be good candidate in this study to be potentially used in the prevention of atherosclerosis-related diseases including CVD.

In this study, radical scavenging activities of *T. crispa* extracts were tested using a methanolic solution of the stable free radical (DPPH) which is known to be stable but sensitive to light, oxygen, pH and type of solvent used (Ozcelik *et al.*, 2003). DPPH radical scavenging activities of TCAE and TCME were quantified for their ability to donate an electron and reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. DPPH radical scavenging assay of *T. crispa* extracts showed that TCAE and TCME posseses a high DPPH radical scavenging activity with DPPH value of 82±1.78 and 73±1.01%, respectively (Table 1). Although, DPPH radical scavenging activity for TCAE and TCME were not comparable to BHT and vitamin C, the antioxidant activity of both extract can be considered as high. Components such as apigenin, picroretoside, berberine, palmatine, picroretine and resin identified from this plant (Kalsom and Noor, 1995) were believed to contribute for this powerful antioxidant capacity.

FRAP assay that has been performed showed FRAP values of TCME and TCAE were not significantly different ($p<0.05$) between each other (Table 1). FRAP value of TCAE and TCME were 1.04±0.27 and 1.64±0.06 mmol $L^{-1}$, respectively. FRAP assay is one of the methods that was used to evaluate antioxidant capacity to measure the ability of the antioxidants to reduce ferric iron ($Fe^{3+}$) to ferrous iron ($Fe^{2+}$). Antioxidants in the samples reduce Fe (III)-TPTZ to form a blue colored Fe (II)-TPTZ complex which results in an increase in the absorbance at 593 nm (Benzie and Strain, 1996). Result of this study showed that both TCAE and TCME have the ability to reduce $Fe^{3+}$ to $Fe^{2+}$. However, its activity was about 2.5 fold lower than vitamin C.

The antioxidant activity in the extract could be explained with the fact that they contained flavonoids and polyphenols as being shown in Table 1. The result demonstrated that the total flavonoids content were not significantly different ($p<0.05$) between extracts but TCME fraction showed a significant higher ($p<0.05$) phenolic content compared to TCAE fraction. Phenolic compounds such as flavonoids, phenolic acids and tannins are considered as antioxidant source of plants. Previous study done by the group has found catechin, luteolin, morin and rutin in *T. crispa* stem extract (Amom *et al.*, 2009).

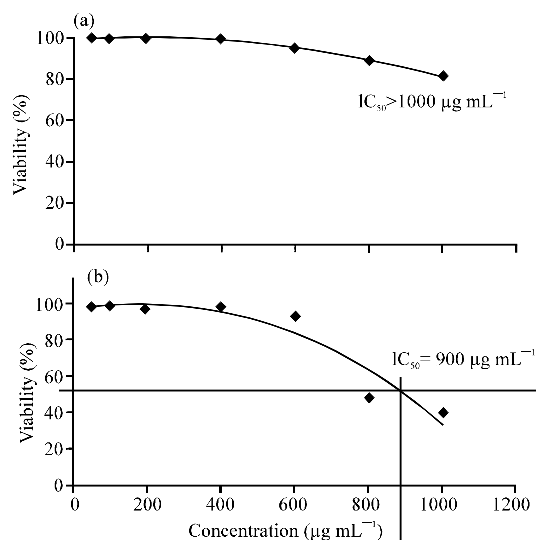


Fig. 1: Viability of HUVECs against; a) TCAE and b) TCME. Data were expressed as mean±SD

These antioxidants also have been reported to have diverse biological activities such as anti-inflammatory and anti-carcinogenic *in vitro* and *in vivo* (Chung *et al.*, 1998). MTT assay of TCAE and TCME on HUVECs performed in this study showed that the median Inhibitory Concentration ($IC_{50}$) of TCME was 900 µg $mL^{-1}$ while $IC_{50}$ of TCAE was exceed 1000 µg $mL^{-1}$ (Fig. 1).

These results showed that TCAE and TCME were not toxic to HUVECs up to concentration 900 µg $mL^{-1}$. The effective concentrations ($EC_{50}$) of both *T. crispa* extracts in attenuating the oxidative effect of $H_2O_2$ were also evaluated.

Both TCAE and TCME-supplemented HUVECs were allowed to settle in the medium for 1 h before the HUVECs were treated with 250 µM of $H_2O_2$, the concentration which can cause 50% death of HUVECs population (Kamal *et al.*, 2009). This experiment showed that TCAE can attenuate the effect of $H_2O_2$ lipid

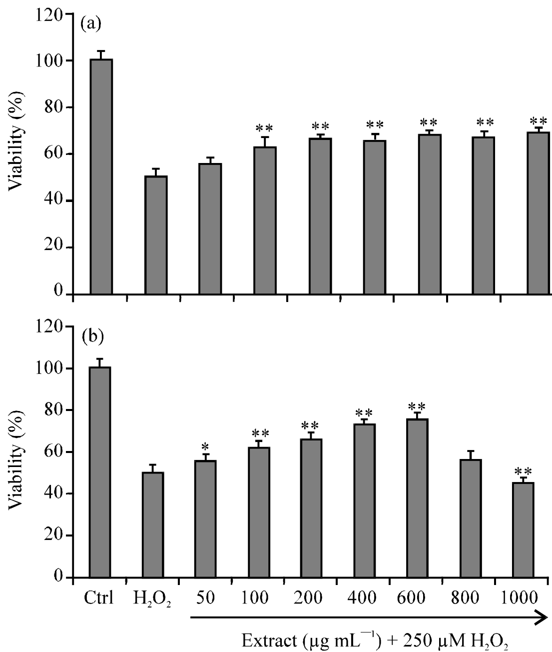


Fig. 2: Protective effect of; a) TCAE and b) TCME on $H_2O_2$-induced HUVECs. Data were expressed as mean±SD. Significantly different against $H_2O_2$ treated alone *p<0.05, **p<0.01

peroxidation in dose-dependent manner (Fig. 2). However, protective effect of TCME on $H_2O_2$-induced HUVECs is only until 600 µg mL$^{-1}$ concentration. The reduction of HUVECs viability after that concentration was probably due to the cytotoxic effect of the TCME itself rather than the cytotoxic effect of $H_2O_2$. A balance between growth and death of endothelial cells is important for the integrity of the vascular endothelium. Although, the mature endothelium *in vivo* is remarkably quiescent, endothelial cells can be activated to grow for purposes of repair following traumatic injury, inflammation, tumor formation or in response to physiological stimuli (Ling *et al.*, 2008). It has been demonstrated that antioxidant enzymes such as CAT, SOD and GPx can prevent living cells membrane from damage and lipid peroxidation (Halliwell, 2005). These antioxidant enzymes play a pivotal role in preventing cellular damage caused by ROS (Luo and Xia, 2006) which will eventually led to atherosclerosis (Schwenke, 1998).

Results of CAT, SOD and GPx assay showed both TCAE and TCME attenuated the changes of antioxidant enzymes expressed on HUVECs which were induced with oxidative agent, $H_2O_2$ (Fig. 3-5). Positive Control (PC) wells in which the HUVECs were exposed to $H_2O_2$ without the treatment of extracts showed the decrease of CAT, SOD and GPx activity compared to Normal Control (NC).

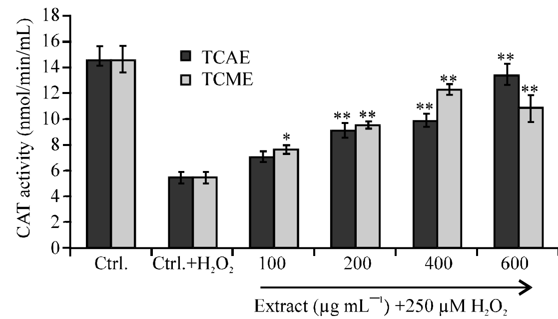


Fig. 3: CAT activity of TCME and TCAE. Data were expressed as mean±SD. Significantly different against $H_2O_2$ treated alone *p<0.05, **p<0.01

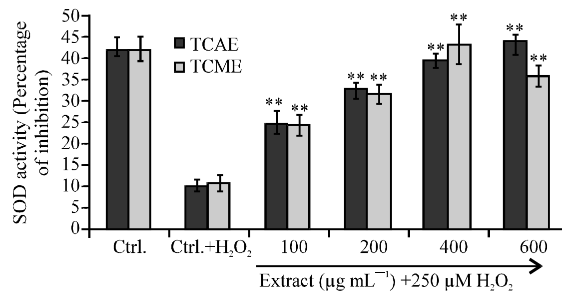


Fig. 4: SOD activity of TCME and TCAE. Data were expressed as mean±SD. Significantly different against $H_2O_2$ treated alone *p<0.05, **p<0.01

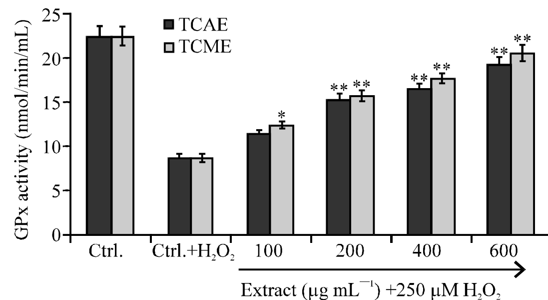


Fig. 5: GPx activity of TCME and TCAE. Data were expressed as mean±SD. Significantly different against $H_2O_2$ treated alone *p<0.05, **p<0.01

This result was in agreement with study that has been performed by Lin *et al.* (2006) which showed that the activity of antioxidant enzymes activity were reduced when HUVECs have been exposed with 250 µM of $H_2O_2$. Compared to PC group, both TCAE and TCME significantly attenuate the effect of $H_2O_2$ by increasing the CAT, SOD and GPx activity on HUVECs treated with $H_2O_2$. These results showed that both TCAE and

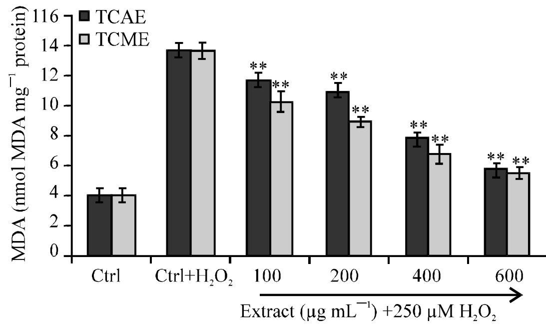


Fig. 6: MDA values of TCAE and TCME. Data were expressed as mean±SD. Significantly different against $H_2O_2$ treated alone *p<0.05, **p<0.01

TCME are able to protect HUVECs from $H_2O_2$ induced injury through anti-oxidative approach. For some years, lipid peroxidation of endothelial cell has been accorded crucial roles in the process of atherosclerosis. Lipid peroxidation is one of the primary events in free radical-mediated endothelial dysfunction and cell injury (Shi *et al*., 2006). MDA is a by product of lipid peroxidation induced by excessive ROS and is widely used as a biomarker of oxidative stress (Cini *et al*., 1994). In present study, the exposure of lipid peroxide such as $H_2O_2$ caused an elevation of MDA level on HUVECs. As shown in the Fig. 6, MDA level has increased upon the incubation of $H_2O_2$ on HUVECs (PC). However, both TCAE and TCME showed a protective effect against $H_2O_2$ on HUVECs which showed an elevated level of MDA. Pre-treatment of TCAE and TCME on HUVECs caused a reduction on MDA level in dose dependent manner for both extracts. These results showed both TCAE and TCME have the ability to reduce MDA level in $H_2O_2$ induced lipid peroxidation on HUVECs.

## CONCLUSION

The present study has elucidated the ability of both TCAE and TCME in preventing the early event of atherosclerosis by antioxidant pathway. Both extracts possess powerful antioxidant as described by TFC, TPC, DPPH and FRAP assay. Both extracts also have been identified to attenuate the effect of $H_2O_2$ on the level of expression of CAT and SOD as well as MDA level of lipid peroxidation.

## REFERENCES

Amom, Z., H. Bahari, S. Isemaail, N.A. Ismail, Z.M. Shah and M.S. Arsyad, 2009. Nutritional composition, antioxidant ability and flavonoids content of *Tinospora crispa* stem. Adv. Nat. Applied Sci., 3: 88-94.

Benzie, I.F.F. and J.J. Strain, 1996. The Ferric Reducing Ability of Plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Anal. Biochem., 239: 70-76.

Bowie, A.G. and L.A. O'Neill, 2000. Vitamin C inhibits NF-κB activation by TNF via the activation of p38 mitogen-activated protein kinase. J. Immunol., 165: 7180-7188.

Chung, K.T., T.Y. Wong, C.I. Wei, Y.W. Huang and Y. Lin, 1998. Tannins and human health: A review. Crit. Rev. Food Sci. Nutr., 38: 421-464.

Cini, M., R.G. Fariello, A. Bianchetti and A. Moretti, 1994. Studies on lipid peroxidation in the rat brain. Neurochem. Res., 19: 283-288.

Fennell, J.P., M.J. Brosnan, A.J. Frater, C.A. Hamilton and M.Y. Alexander *et al*., 2002. Adenovirus-mediated overexpression of extracellular superoxide dismutasimproves endothelial dysfunction in a rat model of hypertension. Gene Ther., 9: 110-117.

Glass, C.K. and J.L. Witztum, 2001. Atherosclerosis: The road ahead. Cell, 104: 503-516.

Halliwell, B., 2005. Free radicals and other reactive species in disease. eLS, 10.1038/npg.els.0003913.

Hollman, P.C. and M.B. Katan, 1999. Dietary flavonoids: Intake, health effects and bioavailability. Food Chem. Toxicol., 37: 937-942.

Hsieh, C.L., G.C. Yen and H.Y. Chen, 2005. Antioxidant activities of phenolic acids on ultra-violet radiation-induced erythrocyte and low density lipoprotein oxidation. J. Agric. Food Chem., 53: 6151-6155.

Kalsom, U. and Y.H. Noor, 1995. Flavone O-glycosides from *Tinospora crispa*. Fitoterapia, 66: 280-280.

Kamal, N.H., A. Zulkhairi, A.H. Hafizah, F. Fazali and A.K.K. Kamilah *et al*., 2009. *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. Res. J. Biol. Sci., : 1230-1235.

Kotze, A.C., 2003. Catalase induction protects *Haemonchuscontortus* against hydrogen peroxide *in vitro*. Int. J. Parasitol., 33: 393-400.

Lin, R., W.R. Wang, J.T. Liu, G.D. Yang and C.J. Hana, 2006. Protective effect of tanshinone IIA on human umbilical vein endothelial cell injured by hydrogen peroxide and its mechanism. J. Ethnopharmacol., 108: 217-222.

Ling, S., L. Nheu, A. Dai, Z. Guo and P. Komesaroff, 2008. Effect of four medicinal herbson human vascular endothelial cells in culture. Int. J. Cardiol., 128: 350-358.

Luo, T. and Z.A. Xia, 2006. Small dose of hydrogen peroxide enhances tumor necrosis factor alpha toxicity in inducing human vascular endothelial cell apoptosis: Reversal with propofol. Anesthesia Analgesia, 103: 110-116.

Malek, A.M. and S. Izumo, 1994. Molecular aspects of signal transduction of shear stress in the endothelial cell. J. Hypertens., 12: 989-999.

Moriue, T., J. Igarashi, K. Yoneda, K. Nakai, H. Kosaka and Y. Kubota, 2008. Sphingosine 1-phosphate attenuates $H_2O_2$-induced apoptosis in endothelial cells. Biochem. Biophys. Res. Commun., 368: 852-857.

Mosmann, T., 1983. Rapid colorimetry assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.

Noor, H. and S.J. Ashcroft, 1998. Pharmacological characterisation of the antihyperglycaemic properties of *Tinospora crispa* extract. J. Ethnopharmacol., 62: 7-13.

Ohkawa, H., N. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95: 351-358.

Ozcelik, O., J.H. Lee and D.B. Min, 2003. Effects of light, oxygen and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. J. Food Sci., 68: 487-490.

Praman, S., M.J. Mulvany, Y. Allenbach, A. Marston, K. Hostettmann, P. Sirirugsa and C. Jansakul, 2011. Effects of an n-butanol extract from the stem of *Tinospora crispa* on blood pressure and heart rate in anesthetized rats. J. Ethnopharmacol., 133: 675-686.

Pratico, D., R.K. Tangirala, D.J. Rader, J. Rokach and G.A. Fitz-Gerald, 1998. Vitamin E suppresses isoprostane generation *in vivo* and reduces atherosclerosis in ApoE-deficient mice. Nat. Med., 4: 1189-1192.

Prior, R.L. and G. Cao, 2000. Flavonoids: Diet and health relationships. Nutr. Clin. Care, 3: 279-288.

Rahman, N.N.N.A., T. Furuta, S. Kojima, K. Takane and M.A. Mohd, 1999. Antimalarial activity of extracts of Malaysian medicinal plants. J. Ethnopharmacol., 64: 249-254.

Schwenke, D.C., 1998. Antioxidants and atherogenesis. J. Nut. Biochem., 9: 424-445.

Shi, G.F., L.J. An, B. Jiang, S. Guan and Y.M. Bao, 2006. Alpinia protocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. Neurosci. Lett., 403: 206-210.

Sulaiman, M.R., Z.A. Zakaria and R. Lihan, 2008. Antinociceptive and antiinflammatory activities of *Tinospora crispa* in various animal model. Int. J. Trop. Med., 3: 66-69.

Velioglu, Y.S., G. Mazza, L. Gao and B.D. Oomah, 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J. Agric. Food Chem., 46: 4113-4117.

WHO, 2003. The world health report 2003. World Health Organization, Geneva, Switzerland.

Yen, G.C. and C.L. Hsieh, 1998. Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models *in vitro*. J. Agric. Food Chem., 46: 3952-3957.

Zakaria, Z.A., A.M. Mat Jais, E.F.P. Henie, H. Zaiton, M.N. Somchit, M.R. Sulaiman and F.O. Faizal, 2006. The *in vitro* antibacterial activity of *Tinosprora crispa* extracts. J. Boil. Sci., 6: 398-401.

Zhishen, J., T. Mengcheng and W. Jianming, 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64: 555-559.