UNIVERSITI PUTRA MALAYSIA

IN VITRO STUDY OF AQUEOUS AND METHANOL EXTRACTS OF *TINOSPORA CRISPA* (L.) HOOK. F. & THOMSON IN EARLY STAGE ATHEROGENESIS

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By

IHSAN SAFWAN BIN KAMARAZAMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2012

DEDICATION

I dedicate this work for all of my family, especially to my mother, Sabariah Bt. Ismail, my father, Kamarazaman B. Abd. Rahman and my wife, Solehatun Bt. Mhd. Bani, for giving me inspiration to pursue my study in this field. Special appreciation to my supervisor, Professor Dr. Zulkhairi Hj. Amom who contributed greatly for my career development and personal growth. Last but not least, I would like to express my appreciation to my research group members, Daryl, Kamal, Amalina, Fazali, Sakinah, Khairunnur Fairuz, Nawal and Jalil and all colleagues who were involved in this project.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master Science

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Chairman : Zulkhairi bin Haji Amom, PhD

Faculty : Medicine and Health Sciences

Tinospora crispa, locally known as 'Patawali' in Malaysia, is a plant belonging to the family of Menispermaceae. It 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 and reported to have antidiabetic, hypotensive and anti-inflammatory activity. The aimed of this study was to investigate the antioxidant properties of this plant as well as its ability to attenuate the release of oxidant and inflammatory markers in induced oxidation and inflammation in human umbilical vein cells (HUVECs).

In vitro studies have been conducted to evaluate antioxidant properties of *T. crispa*. The radical scavenging activity was tested by 1,1-diphenyl-2-picrylhydraxyl (DPPH) assay. The result showed DPPH scavenging activity of *T. crispa* aqueous (TCAE) and methanol extract (TCME) were 82 ± 1.78 and 73 ± 1.01%, respectively. The ability of *T. crispa* extracts to reduce Fe^{3+} to Fe^{2+} were tested by FRAP assay and the result showed FRAP value of TCAE and TCME were 1.04 ± 0.27 and 1.64 ± 0.06 mmol/L, respectively. Total flavonoids content (TFC) and total phenolics content (TPC) were also measured. The result showed TFC value of TCAE and TCME were 205.58 ± 3.5 and 223 ± 10.49 mg QE/g sample, respectively while TPC value of TCAE and TCME were 32.58 ± 0.68 and 41.64 ± 0.97 mg GAE/mg sample, respectively.

The antioxidant enzymes activities and the level of anti-inflammatory markers in HUVECs treated with TCAE and TCME to counter the oxidative effect by hydrogen peroxide (H_2O_2) or inflammatory effect by tumor necrosis factor- α (TNF-α) were also measured. HUVECs were seeded at 1 x 10^6 cell/well in 6-well plate and the treatments were divided into 3 groups; normal control, negative control, and treated groups. In the negative control (NC) group, HUVECs were exposed to either 250 μM H_2O_2 or 10 ng/mL TNF-α alone, whereas in the treated groups HUVECs were pretreated with various concentrations of TCAE and TCME (100, 200, 400 and 600 μg/mL) for 30 minutes prior to exposure to H_2O_2 (250 μM) or TNF-α (10 ng/mL). In the normal control groups, HUVECs were incubated with culture medium only. The cells were incubated for 24 hours at 37 ^oC with 5% CO_2 supply for further analysis. Assays that were performed in

present study were antioxidant enzyme activities such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), lipid peroxidation level by malondealdehyde (MDA) assay, and inflammatory markers such as nitric oxide (NO), intercellular cell adhesion molecule (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF).

Results of antioxidant enzymes activity assays (CAT, SOD and GPx) showed TCAE and TCME at a concentration ranges from 100-600 µg/ml significantly increased ($p<0.05$) the level of those antioxidant enzymes compared to NC. Results of MDA assay showed significant reduction ($p<0.05$) of MDA level in HUVECs treated with TCAE and TCME compared to NC. Concomitantly, the level of NO expression in HUVECs treated with TCAE and TCME was significantly elevated ($p<0.05$) compared to NC. In addition, inflammatory markers assays showed TCAE and TCME have significantly reduced ($p<0.05$, 0.01) the secretion of ICAM-1, VCAM-1 and M-CSF as compared to NC. However, secretion of MCP-1 was not reduced by the treatment of TCAE and TCME. Taken together, this study suggest that TCAE and TCME can effectively prevent oxidative stress by H_2O_2 and inflammation by TNF-α on HUVECs, which might be importance in the treatment of atherosclerosis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah master Sains

KAJIAN *IN VITRO* EKSTRAK AKUES DAN METANOL *TINOSPORA CRISPA* (L.) HOOK. F. & THOMSON PADA PERINGKAT AWAL ATEROGENESIS

Oleh

IHSAN SAFWAN BIN KAMARAZAMAN

April 2012

Pengerusi : Zulkhairi bin Haji Amom, PhD

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Tinospora crispa, dikenali sebagai 'Patawali' di Malaysia, adalah sejenis tumbuhan tergolong dari famili Menispermaceae. Ia adalah pokok pemanjat yang boleh ditemui di hutan primer di Malaysia, Indonesia, Thailand dan Vietnam. *T. crispa* telah digunakan secara tradisi untuk merawat diabetis, darah tinggi dan sakit belakang. *T. crispa* sebelum ini dilaporkan mempunyai kesan antidiabetik, hipotensif dan anti-inflamasi. Tujuan kajian ini dijalankan adalah untuk mengkaji keupayaan antioksidan tumbuhan ini dan juga keupayaan tumbuhan ini merencat isyarat pengoksidaan dan inflamasi pada sel vena endothelial umbilikal manusia (HUVECs) teraruh.

Kajian *in vitro* telah dijalankan untuk menilai kesan antioksidan oleh *T. crispa*. Kesan perambatan radikal dilakukan menggunakan asai DPPH dan keputusan

ujian ini menunjukkan kesan perambatan radikal DPPH oleh ekstrak akues *T. crispa* (TCAE) adalah 82 ± 1.78% dan 73 ± 1.01% bagi ekstrak metanol (TCME). Keupayaan ekstrak *T. crispa* untuk menurunkan Fe^{3+} kepada Fe^{2+} diuji dengan asai FRAP dan keputusannya menunjukkan nilai FRAP bagi TCAE adalah 1.04 ± 0.27 mmol/L manakala nilai FRAP bagi TCME adalah 1.64 ± 0.06 mmol/L. Kandungan flavonoid total (TFC) dan kandungan fenolik total (TPC) juga dikaji. Keputusan kajian ini menunjukkan nilai TFC bagi TCAE adalah sebanyak 205.58 ± 3.5 QE/g sampel manakala nilai TPC bagi TCME adalah sebanyak 223 ± 10.49 mg QE/g sampel. Nilai TPC bagi TCAE adalah sebanyak 32.58 ± 0.68 mg GAE/mg sampel manakala nilai TPC bagi TCME adalah sebanyak 41.64 ± 0.97 mg GAE/mg sampel.

Kajian ini juga menilai akitiviti enzim antioksidan dan aras penghasilan isyarat anti-inflamasi pada sel HUVEC yang dirawat TCAE dan TCME terhadap kesan pengoksidaan oleh hidrogen peroksida (H_2O_2) dan inflamasi oleh faktor nekrosis tumor-α (TNF-α). HUVEC sebanyak 1 x 10^6 dikultur pada plat 96 telaga dan rawatan dibahagikan kepada 3 kumpulan iaitu kumpulan kawalan normal, kumpulan kawalan negatif (NC) dan juga kumpulan rawatan. Bagi kumpulan kawalan negatif, HUVEC hanya dirawat samada H_2O_2 dengan kepekatan 250 µM atau TNF-α dengan kepekatan 10 ng/ml manakala bagi kumpulan rawatan, HUVEC dirawat dengan TCAE atau TCME pada kepekatan 100, 200, 400 dan 600 µg/ml selama 30 minit sebelum oleh samada kepada H_2O_2 (250 µM) atau TNF-α (10 ng/ml). Bagi kumpulan normal pula, hanya mempunyai HUVEC. Sel kemudian dieram dalam inkubator selama 24 jam pada 37 ^oC dengan

kehadiran 5% CO_2 bagi analisis seterusnya. Asai yang dilakukan dalam kajian ini ialah asai enzim antoksidan seperti catalase (CAT), superoxide dismutase (SOD) dan glutathione peroxide (GPx), asai peroksidaan lipid iaitu asai malondealdehyde (MDA) dan juga asai inflamasi seperti nitrik oksida (NO), *intercellular cell adhesion molecule-1* (ICAM-1), *vascular cell adhesion molecule-1* (V-CAM-1), *monocyte chemotactic protein-1* (MCP-1) dan *macrophage cell stimulating factor* (M-CSF).

Bagi asai aktiviti enzim antioksidan (CAT, SOD dan GPx), keputusan ujian ini menunjukkan TCAE dan TCME pada kepekatan antara 100-600 µg/ml telah menyebabkan peningkatan yang ketara ($p<0.05$) bagi aras enzim antioksida tersebut. Bagi asai MDA pula, keputusan ujian ini menunjukkan penurunan ketara ($p<0.05$, 0.01) bagi aras MDA dalam HUVEC yang dirawat oleh TCAE dan TCME berbanding NC. Pada masa yang sama, penghasilan NO dalam HUVEC yang dirawat oleh TCAE dan TCME meningkat secara ketara ($p<0.05$) berbanding NC. Selain itu, isyarat inflamasi menunjukkan TCAE dan TCME telah menyebabkan penurunan signifikan ($p<0.05$, 0.01) pada aras penghasilan ICAM-1, VCAM-1 dan M-CSF berbanding NC. Walaubagaimanapun, penghasilan MCP-1 tidak berkurangan dengan rawatan TCAE dan TCME. Secara keseluruhannya, kajian ini menunjukkan bahawa TCAE dan TCME boleh melindungi sel HUVEC daripada kesan pengoksidaan oleh H_2O_2 dan juga kesan inflamasi oleh TNF-α, menunjukkan kegunaannya dalam rawatan aterosklerosis.

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I certify that an Examination Committee has met on 5 April 2012 to conduct the final examination of Ihsan Safwan Bin Kamarazaman on his Master thesis entitle *In Vitro* Study of Aqueous and Methanol Extracts of *Tinospora Crispa (L.)* Hook. *F*. & Thomson in Early Stage Atherogenesis in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the Master of Science. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or any other institutions.

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IHSAN SAFWAN BIN KAMARAZAMAN

Date: 5 April 2012

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