



UNIVERSITI PUTRA MALAYSIA

***ANTI-HYPERPERMEABILITY EFFECT OF MALAYSIAN TUALANG
HONEY ON HYDROGEN PEROXIDE-INDUCED INCREASED VASCULAR
PERMEABILITY***

KOGILAVANEE DEVASVARAN

FPSK(m) 2016 58



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By

KOGILAVANEE DEVASVARAN

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

November 2016

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

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KOGILAVANEE DEVASVARAN

November 2016

Chairman : Yong Yoke Keong, PhD
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Vascular hyperpermeability remains the main cause of underlying disorders in myocardial ischemia and atherosclerosis. Tualang Honey (TH) has been used in traditional medicine for decades and proven to possess multiple pharmacological actions. However to date, little is known about the use of TH in anti-inflammatory activity specifically in endothelial barrier protection. Thus, this study aimed to investigate the effects of TH on H₂O₂-induced endothelial hyperpermeability *in vitro* and supported by an *in vivo* approach. In order to determine the effect of TH on endothelial hyperpermeability, HUVEC was pre-treated with pre-defined non-cytotoxic concentration (via cytotoxicity assay) of TH for 4h and then exposed to 0.5mM H₂O₂. FITC-dextran was used as a permeability indicator. The supportive *in vivo* study was the Miles assay, which quantified the extravasation of Evans blue dye in the dorsal skin of balb/c mice. To examine the cells morphological alterations, adherence junction proteins in HUVEC were identified using Fluorescein Phalloidin, caveolin-1 and β -catenin immunofluorescence labeling. Intracellular calcium, PKC and cAMP signaling were also investigated. All data was analyzed using SPSS. LD₅₀ of TH was found to be 3.7% and concentrations ranging from 0.01% -1% showed no cytotoxic effect to HUVEC. Induction with H₂O₂ was found to increase HUVEC permeability but the effect was significantly reversed by TH ($p<0.05$), of which the permeability inhibition peaked at 0.1%. TH also significantly ($p<0.05$) reduced the effect of H₂O₂ *in vivo* in all concentrations in a dose-dependent manner. Immunofluorescence confirmed that TH reduced stress fiber formation and the co-localization of caveolin-1 and β -catenin in HUVEC. TH also significantly ($p<0.05$) decreased intracellular calcium release and PKC activity, while maintained the level of cAMP when induced with H₂O₂. In conclusions, TH ameliorates H₂O₂-induced endothelial hyperpermeability *in vitro* and *in vivo* via suppression of adherence

junction protein re-distribution, reduction of intracellular calcium and PKC activity while maintaining the cAMP production.



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

KESAN ANTI-KEBOLEHTELAPAN MADU TUALANG MALAYSIA KE ATAS HIDROGEN PEROKSIDA TERARUH YANG MENINGKATKAN KEBOLEHTELAPAN VASKULAR

Oleh

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Hiperkebolehtelapan vaskular kekal sebagai punca utama gangguan iskemia miokardium dan aterosklerosis. Madu Tualang (TH) telah digunakan dalam perubatan tradisional selama beberapa dekad dan terbukti mempunyai pelbagai tindakan farmakologi. Namun setakat ini, hanya sedikit yang diketahui tentang penggunaan TH dalam aktiviti anti-radang khususnya dalam perlindungan halangan endotelial. Oleh itu, kajian ini bertujuan untuk mengkaji kesan TH pada H₂O₂ teraruh hiperkebolehtelapan endotelial secara “*in vitro*” dan disokong dengan pendekatan “*in vivo*”. Bagi menentukan kesan TH ke atas hiperkebolehtelapan endotelial, HUVEC dipelihara dengan kepekatan bukan sitotoksik yang telah ditetapkan (melalui cerakin sitotoksik) TH selama 4 jam dan kemudiannya didedahkan kepada 0.5mM H₂O₂. FITC-dextran telah digunakan sebagai petunjuk kebolehtelapan. Sokongan dalam kajian “*in vivo*” ialah cerakin Miles, yang diukur dengan pengeluaran pewarna biru Evans pada kulit belakang mencit balb/c. Bagi mengkaji perubahan morfologi sel, kelekapan protein simpang di HUVEC telah dikenal-pasti dengan menggunakan Fluorescein Phalloidin, kaveolin-1 dan pelabelan β-katenin immunopendaflour. Kalsium intraselular, PKC dan isyarat cAMP juga telah dikaji. Semua data dianalisa dengan menggunakan SPSS. LD₅₀ TH ialah sebanyak 3.7% dan kepekatan berjulat dari 0.01% -1% tidak menunjukkan kesan sitotoksik kepada HUVEC. Aruhan dengan H₂O₂ didapati meningkatkan kebolehtelapan HUVEC tetapi kesannya adalah jauh bertentangan dengan TH ($p < 0.05$), iaitu perencatan kebolehtelapan memuncak pada 0.1%. TH juga mengurangkan kesan *in vivo* H₂O₂ secara signifikan ($p < 0.05$) bagi semua kepekatan dalam berkeperluan dos. Immunopendaflour mengesahkan bahawa TH mengurangkan pembentukan serat tekanan dan penempatan bersama kaveolin-1 dan β-katenin dalam HUVEC. TH juga mengurangkan pengeluaran kalsium intraselular dan aktiviti PKC secara signifikan ($p < 0.05$), di samping mengekalkan tahap cAMP apabila diaruh dengan H₂O₂. Sebagai kesimpulannya, TH merendahkan aruhan H₂O₂ hiperkebolehtelapan endotelial secara “*in vitro*” dan “*in vivo*” melalui

perencatan pengagihan semula kelebihan protein simpang, penurunan kalsium intraselular dan aktiviti PKC di samping mengekalkan pengeluaran cAMP.



ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Dr. Yong Yoke Keong for his full support, expert guidance, understanding and encouragement throughout my study and research. Without his incredible patience and timely wisdom and counsel, my thesis work would have been a frustrating and overwhelming pursuit. In addition, I express my appreciation to Dr. Tan Jun Jie and the late Assoc. Prof. Dr. Zuraini Ahmad for having served on my committee. Their thoughtful questions and comments were valued greatly.

I would also like to thank all the post-graduate students and staffs of the Department of Human Anatomy, Faculty of Medicine and Health Sciences, UPM for their help throughout my study. Special thanks go to my few friends who helped and supported me during my study and writing of the thesis.

Finally, I would like to thank my parents, siblings and close family members for their unconditional love and support during the last two years. I would not have been able to complete this thesis without their continuous love and encouragement.

I certify that a Thesis Examination Committee has met on 7 November 2016 to conduct the final examination of Kogilavane a/p Devasvaran on her thesis entitled "Anti-Hyperpermeability Effect of Malaysian Tualang Honey on Hydrogen Peroxide-Induced Increased Vascular Permeability" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

$\cdot\text{O}_2^-$	Superoxide anion
OH	Hydroxyl radical
AC	Adenylyl cyclase
AJ	Adherence Junctions
ANG-1	Angiopoetin-1
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
CAV-1	Caveolin-1
CAV-2	Caveolin-2
CAV-3	Caveolin-3
CVD	Cardiovascular Disease
DAG	1,2-diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCF	2'-7'-Dichlorofluorescin
DCFDA	2',7'-Dichlorofluorescin Diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme Linked Immuno-Sorbent Assay
FITC	Fluorescein isothiocyanate
GC-MS	Gas chromatography–mass spectrometry
H ₂ O ₂	Hydrogen peroxide
HPLC	High performance liquid chromatography

HUVEC	Human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
IEJ	Interendothelial junctions
IJN	Institute Jantung Negara
IP3	Inositol 1, 4, 5-trisphosphate
JAM	Junctional adhesion molecules
LC50	Lethal Concentration
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MGO	Methylglyoxal
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PBS	Phosphate buffer saline
pH	Potential hydrogen
PKA	Cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phosphoinositide-specific phospholipase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.E.M	Standard error of mean
SIP	Sphingosine-1-phosphate
SPSS	Statistical Package for Social Sciences
TH	Tualang Honey
TJ	Tight Junctions

TNF- α	Tumor necrosis factor-alpha
TR	Trolox
TRITC	Tetramethylrhodamine
UK	United Kingdom
USA	United State of America
VEGF	Vascular endothelial growth factor
VPF	Vascular penetrability component
WHO	World Health Organisation



CHAPTER 1

INTRODUCTION

1.1 Background

The number one cause of death worldwide is cardiovascular diseases (CVDs). More people are dying from it each year than from any other illness. Based on the records by the World Health Organization (WHO), 17.5 million people, which represents a total of 31% died from CVDs in 2012. Estimated around 7.4 million from this deaths were due to coronary heart disease meanwhile, 6.7 million deaths were caused by stroke. CVDs can be defined as disorders related to the heart and blood vessels or also known as the vascular system.

The vascular system comprises of the body's network of blood vessels, which are classically known as the arteries, veins and capillaries. The function of this system is to carry blood to and from the heart (Mehta and Malik, 2006). The inner most layer of the blood vessel is known as the tunica intima, which is where the endothelial cell monolayer integrity is maintained via the control of vascular homeostasis. An alteration in the endothelial integrity alters vascular permeability, usually in conditions of inflammation, diseases, and atherosclerosis (Mehta and Malik, 2006).

Reactive oxygen species (ROS) are produced in normal cellular metabolism but are highly unstable due to its incomplete reduction of molecular oxygen. ROS at physiological levels, plays a dynamic role in modulating several signaling pathways, related to cell differentiation and growth (Sauer et al., 2001). Furthermore, ROS overproduction can induce oxidative stress which is due to an imbalance in the levels favouring oxidants over antioxidants which stimulates the loss of cell-cell adhesion leading to an increase in the paracellular permeability (Mehta and Malik, 2006). This eventually causes endothelial damage which promotes various vascular disorders, such as heart failure, diabetes mellitus, atherosclerosis, dyslipidaemia, myocardial infarction, angina pectoris and hypertension (Schächinger et al., 2000; Neunteufl et al., 2000; Heitzer et al., 2001; Gokce et al., 2002). Thus, it is crucial to study a preventative medicine to suppress the effects of oxidative stress which could prevent disease progression.

Tualang Honey (TH) is a therapeutic honey present in the honeycombs of the *Koompassia excels* (Tualang Tree), which is found in the tropical rainforest in Malaysia. This particular honey is currently being widely studied for its beneficial properties, including promoting wound healing and antibacterial effects (Nasir et al., 2010; Khoo et al., 2010). Besides, TH has been accounted for having a high ascorbic acid, flavonoid and phenolic content, which in turn contributes to its potent antioxidant activity (Khalil et al., 2012; Kishore et al., 2011). Although numerous studies were conducted on the positive effects exhibited by TH, the potential of TH

in the study of HUVEC permeability has not been investigated. Thus, this study aims to investigate the possible anti-hyperpermeability effects of Malaysian TH in H₂O₂-induced HUVEC cells.

1.2 Problem Statement

Oxidative stress is a major contributor to most vascular system related diseases, in particular vascular hyperpermeability. The endothelium which is found in the innermost layer of blood vessels has an essential role in mediating vascular related diseases. To date there are only a few barrier stabilizing mediators that has been reported, which are the cyclic adenosine monophosphate (cAMP), sphingosine-1-phosphate (SIP) and angiotensin-1 (Ang-1). These mediators may itself contribute to adverse effects if exposed as an independent molecule. Thus, it is important to identify natural medicines, which are readily available to act as a barrier stabilizing agent to prevent or combat vascular related diseases with no known side effects.

1.3 Significance of the Study

This study was conducted to identify the role of TH and its barrier stabilizing activities exhibited upon induction of oxidative stress in the endothelial barrier. TH acts as a preventative medicine and is able to suppress the effects of H₂O₂-induced increased vascular hyperpermeability.

Since TH showed a potent anti-oxidant/anti-inflammatory effect on the endothelial barrier, it could be suggested that the use of TH may be able to promote a healthy lifestyle. Apart from that, the society would benefit from an anti-inflammatory/anti-oxidant derived from nature as there is no reported side effect. The economy would also improve as TH is found in Malaysia, and more bee farms could be implemented which opens job opportunities to our local citizens.

Thus, TH could alleviate the possibility of developing vascular related diseases affecting millions of people and at the same time improve Malaysia's economy by improving the work force.

1.4 Hypothesis

TH will suppress increased vascular permeability induced by H₂O₂ *in vivo* and *in vitro* via the suppression of; actin cytoskeleton reorganization, localization of β - catenin from caveolin-1 (cav-1), reduction of intracellular calcium influx and Protein Kinase C (PKC) activities, despite sustaining the cAMP level.

1.5 Objective of the Study

1.5.1 General Objective

To determine the effects of TH on H₂O₂-induced vascular hyperpermeability *in vivo* and *in vitro*.

1.5.2 Specific Objective

1. To investigate the effect of TH on H₂O₂-induced vascular hyperpermeability in mice and HUVEC cells.
2. To examine the effect of TH on the vascular endothelium morphology in term of actin cytoskeleton induced by H₂O₂.
3. To determine the effect of TH on vascular endothelium adherence junctions induced by H₂O₂.
4. To elucidate the mechanism of action of TH by studying the intracellular calcium and PKC activity.
5. To study the effect of TH on maintaining vascular endothelium barrier function via cAMP.

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BIODATA OF STUDENT

My name is Kogilavane Devasvaran and I am the fifth/youngest child in my family. I was born on May 16th in Malacca but was raised in Johor throughout my life, where I schooled at Sekolah Kebangsaan Kulai 2 during my primary level and furthered my secondary education at Sekolah Menengah Kebangsaan Bandar Putra, Kulai, Johor. Then, I enrolled in Form 6, under the biology field in Sekolah Menengah Kebangsaan Sultan Ismail, Kulai. From here I begun to have passion towards biology and thus enrolled in a Bachelor in Biomedicine (Hons) programme in Management and Science University (MSU).

During my internship, I was exposed to neuronal cell culture which made me develop an interest towards the study of cells. Therefore, I decided to further my studies in a Master project which relates me to cellular physiology. I then enrolled in UPM and started my master project on endothelial cell culture in 2014. In future, I would like to pursue my PhD in cellular studies to gain more knowledge and experience as a researcher.

PUBLICATION

Devasvaran, K., & Yong, Y. K. (2016). Anti-inflammatory and wound healing properties of Malaysia Tualang honey. *Curr Sci*, 110(1), 47-51.





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