UNIVERSITI PUTRA MALAYSIA

IN VITRO PROTECTIVE EFFECTS OF 2,4,6-TRIHYDROXY-3-GERANYL ACETOPHENONE ON JUNCTIONAL PROTEIN DISRUPTION AND THE UNDERLYING SIGNALING PATHWAYS IN LIPOPOLYSACCHARIDE-INDUCED ENDOTHELIAL HYPERPERMEABILITY

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By

CHAN YEE HAN

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

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Endothelial hyperpermeability is a prominent hallmark in the pathogenesis of diseases such as sepsis and endotoxemia. Proinflammatory stimuli such as lipopolysaccharide (LPS) could trigger the overproduction of proinflammatory mediators and activate multiple signaling pathways, thereby resulting in junctional protein disruption and endothelial hyperpermeability. As such, inhibiting the structural and functional impairment of junctional proteins is thought to be crucial in treating endothelial hyperpermeability. 2,4,6-Trihydroxy-3-geranyl acetophenone (tHGA) is a bioactive phloroglucinol compound found in the young leaves of *Melicope pteleifolia* (Champ. ex Benth.) T.G.Hartley. Previous study has proven that tHGA exhibited significant *in vitro* barrier protective effects against LPS induction, mainly by inhibiting endothelial hyperpermeability via the attenuation of F-actin cytoskeletal rearrangement. In endothelium, F-actin cytoskeleton is anchored to cell-cell junctional proteins such as zonula occluden (ZO)-1, occludin, and vascular endothelial-cadherin (VE-cadherin), and plays collaborative roles with them in the preservation of endothelial integrity. Therefore, effects of tHGA on these junctional proteins should be further investigated. Also, more in-depth investigations are required to further dissect the signaling pathways mediated by tHGA in suppressing LPS-induced endothelial hyperpermeability. In the present study, Human Umbilical Vein Endothelial Cells (HUVECs) were pretreated with tHGA for 6 hours, followed by LPS induction with respective durations. The cells were divided into seven experimental groups including vehicle control, tHGA control, LPS control, dexamethasone drug control, and tHGA treatment (1.25, 5, and 20 μM) groups. Several parameters related to endothelial hyperpermeability were assessed. Firstly, transendothelial electrical resistance (TEER) assay was performed to examine the effect of tHGA on endothelial junctional integrity during LPS induction. Secondly, immunofluorescence staining was conducted to evaluate the effect of tHGA on the localization of junctional proteins along the cell

periphery. Thirdly, Western Blot and Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) were conducted to elucidate the effect of tHGA on the expression of junctional proteins at protein and gene level, respectively. Lastly, the effect of tHGA on the activation of several proinflammatory signaling molecules such as NF-κB p65, myosin light chain (MLC), p38 MAPK, ERK MAPK, and JNK MAPK were assessed in order to explore the signaling pathways involved in the protective effects of tHGA. The findings demonstrated that tHGA was able to preserve endothelial junctional integrity significantly at 20 μM during LPS induction. Interestingly, all concentrations of tHGA significantly preserved intact ZO-1 and VE-cadherin along the cell periphery, via the abrogation of delocalization, as evidenced by the attenuation of intercellular gap formation. In terms of expression, 5 and 20 μM tHGA exerted significant inhibitory effects against the down-regulation of ZO-1 and VE-cadherin at both protein and gene level. Interestingly, only 20 μM was able to alleviate occludin down-regulation. More in-depth investigations later found that tHGA primarily targeted GEF-H1/RhoA/ROCK pathway in the manifestation of protective effects against LPS-induced junctional protein disruption, as evidenced by its abilities to inhibit the activation of downstream signaling molecules including MLC, NF-κB p65, p38 MAPK, and ERK MAPK. In conclusion, tHGA profoundly abrogated LPS-induced endothelial hyperpermeability by suppressing the delocalization and down-regulation of junctional proteins including ZO-1, occludin, and VE-cadherin, via the inactivation of MLC, NF-κB p65, p38 MAPK, and ERK MAPK, which are mainly diverged from GEF-H1/RhoA/ROCK pathway. As such, it is highly recommended that tHGA should be further developed into a potential therapeutic remedy for treating various disorders related to uncontrolled or prolonged endothelial hyperpermeability.

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

KESAN-KESAN PERLINDUNGAN *IN VITRO* 2,4,6-TRIHYDROXY-3-GERANYL ACETOPHENONE TERHADAP DISFUNGSI PROTEIN SIMPANG DAN JALUR ISYARAT SEL DALAM HIPERKEBOLEHTELAPAN ENDOTELIUM TERARUH LIPOPOLISAKARIDA

Oleh

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Hiperkebolehtelapan endothelium merupakan cirri utama patogenesis penyakit seperti sepsis dan endotoksemia. Rangsangan proinflamasi seperti lipopolisakarida (LPS) boleh menyebabkan penghasilan mediator proinflamasi yang berlebihan dan mengaktifkan pelbagai jalur isyarat sehingga mengakibatkan disfungsi protein simpang dan hiperkebolehtelapan endothelium. Oleh itu, perencatan kerusakan struktur dan fungsi protein simpang dianggap penting untuk merawat kemusnahan endotelium. 2,4,6-Trihydroxy-3-geranyl acetophenone (tHGA) merupakan sebatian phloroglucinol bioaktif yang terdapat pada daun muda *Melicope pteleifolia* (Champ. ex Benth.) T.G.Hartley. Kajian dahulu membuktikan bahawa tHGA menunjukkan kesan perlindungan *in vitro* yang ketara terhadap induksi LPS, khususnya penghalangan hiperkebolehtelapan endotelium melalui perencatan penyusunan semula sitoskeleton F-aktin. Di endotelium, sitoskeleton F-aktin dilekatkan pada protein simpang seperti zonula occluden (ZO)-1, occludin, dan vascular endothelial-cadherin (VE-cadherin). Sitoskeleton F-aktin juga memainkan peranan kerjasama dengan protein simpang untuk memelihara integriti endotelium. Oleh itu, kesan tHGA terhadap protein simpang harus dikaji dengan lebih lanjut. Selain itu, penyelidikan yang lebih mendalam juga diperlukan untuk mengetahui jalur isyarat sel yang dipenggaruhi oleh tHGA dalam pengelakan hiperkebolehtelapan endotelium semasa induksi LPS. Dalam kajian ini, Sel Endotelium Urat Umbilikal Manusia (HUVECs) telah diprarawatkan dengan tHGA selama 6 jam dan didedahkan kepada LPS dengan jangka masa berkenaan. Tujuh kumpulan eksperimen merangkumi kumpulan normal, tHGA, LPS, ubat dexametason, dan rawatan tHGA (1.25, 5 dan 20 μM). Beberapa faktor yang berkaitan dengan hiperkebolehtelapan endotelium telah dinilai. Pertama, ujian rintangan elektrik transendothelial (TEER) dijalankan untuk mengkaji kesan tHGA terhadap integriti simpang endotelium semasa induksi LPS. Kedua, imunofluoresen telah dilakukan untuk menilai kesan tHGA terhadap

penyetempatan protein simpang sepanjang pinggiran sel. Ketiga, Tindak Balas Berantai Polimerase Masa Sebenar (RT-qPCR) dan Western Blot dijalankan masing-masing untuk menjelaskan pengaruhan tHGA terhadap ekspresi protein simpang pada tahap protein dan gen. Akhirnya, kesan tHGA terhadap pengaktifan beberapa molekul isyarat proinflamasi seperti NF-κB p65, myosin light chain (MLC), p38 MAPK, ERK MAPK, dan JNK MAPK telah dinilai untuk mengetahui jalur isyarat sel yang terlibat dalam kesan perlindungan tHGA. Hasil kajian menunjukkan bahawa hanya 20 μM tHGA dapat memelihara integriti simpang endothelium semasa induksi LPS. Semua kepekatan tHGA dapat mengekalkan ZO-1 dan VE-cadherin yang utuh sepanjang pinggiran sel dengan mengelakkan penyusunan semula. Dari segi ekspresi, 5 dan 20 μM tHGA menunjukkan kesan perencatan yang ketara terhadap pengungkapan ZO-1 dan VE-cadherin pada tahap protein dan gene. Namun begitu, hanya 20 μM tHGA mampu mengelakkan pengungkapan occludin. Penyelidikan yang lebih mendalam mendapati bahawa tHGA mensasarkan jalur GEF-H1/RhoA/ROCK dalam pelahiran kesan perlindungan terhadap protein simpang semasa induksi LPS. Hal ini dibuktikan oleh kemampuan tHGA untuk menghalang pengaktifan molekul isyarat seperti MLC, NF-κB p65, p38 MAPK dan ERK MAPK. Kesimpulannya, tHGA dapat mengelakkan hiperkebolehtelapan endotelium semasa induksi LPS dengan menghalang delokalisasi dan pengungkapan protein simpang termasuk ZO-1, occludin dan VE-cadherin. Kemampuan tersebut disumbang oleh penonaktifan molekul cawangan jalur GEF-H1/RhoA/ROCK, iaitu MLC, NF-κB p65, p28 MAPK dan ERK MAPK. Oleh sedemikian, tHGA berpotensi untuk dibangunkan sebagai ubat terapi kepada penyakit yang berkaitan dengan hiperkebolehtelapan yang tidak terkawal atau berpanjangan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisorry Committee were as follow:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS / NOTATIONS

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
ADP	Adenosine diphosphate
AJ	Adherens junction
AKT	Protein kinase B
ANOVA	Analysis of variance
APS	Ammonium persulfate
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BRB	Blood-retina barrier
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CAM	Cell adhesion molecule
CCD	Charged-coupled device
cDNA	Complementary deoxyribonucleic acid
CER I	Cytoplasmic extraction reagent I
CER II	Cytoplasmic extraction reagent I
CO_2	Carbon dioxide
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipase A2
c-Src	Proto-oncogene tyrosine-protein kinase Src
CYP2D6	Cytochrome P450 2D6

CysLT	Cysteinyl leukotriene
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Autoclaved deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
dNTP	Deoxynucleotide triphosphate
E-cadherin	Epithelial-cadherin
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
EVOM	Epithelial volt/ohm meter
F-actin	Filamentous actin
FBS	Fetal bovine serum
G-actin	Globular actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GJ	Gap junction
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanine triphosphate
HCl	Hydrochloric acid
HMVEC-L	Human Lung Microvascular Endothelial Cell
HRP	Horseradish peroxidase

HSVEC	Human Saphenous Vein Endothelial Cell
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IgE	Immonuglobulin E
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IRAK1	Interleukin 1 receptor associated kinase 1
IRAK4	Interleukin 1 receptor associated kinase 4
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
LAT	Linker of activated T cells
LOX	Lipooxygenase
LPS	Lipopolysaccharide
LTC4	Leukotriene C4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD2	Myeloid differentiation factor 2
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

MyD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NER	Nuclear extraction reagent
NF-κB	Nuclear factor-kappa light chain enhancer of activated B cells
NMMII	Non-muscle myosin II
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase Chain Reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PLCγ1	Phosphoinositide-specific phospholipase C gamma 1
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PSA	Passive systemic anaphylaxis
PVDF	Polyvinylidene fluoride
RANTES	Regulated upon activation, Normal T cell expressed and secreted
Rho	Ras homolog family
RhoA	Ras homolog family member A
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid

ROCK	Rho-associated protein kinase
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
S.E.M.	Standard error of mean
SAPK	Stress-activated protein kinase
Scd14	Soluble cluster of differentiation 14
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Ser	Serine
STAT3	Signal transducer and activator of transcription 3
Syk	Spleen tyrosine kinase
TAB	TAK1-binding protein
TAK1	Transforming growth factor-beta-activated kinase 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween 20
TEER	Transendothelial electrical resistance
TEMED	Tetramethylethylenediamine
TF	Tissue factor
TGF-1	Transforming growth factor-1\
tHGA	2,4,6-trihydroxy-3-geranyl acetophenone
Thr	Threonine
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TJ	Tight junction
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
TOPKAT	Toxicity prediction by computer assisted technology

TRAF6	Tumor necrosis factor receptor-associated factor 6
Ubc13	Ubiquitin-conjugating enzyme 13
Uev1A	Ubiquitin-conjugating enzyme variant 1A
VCAM-1	Vascular adhesion molecule-1
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VVO	vesiculo-vacuolar organelle
vWF	von Willebrand factor
ZO	Zonula Occluden
3-GAP	3-geranyl-2,4,6-trihydroxyacetophenone

Notations

α	Alpha
β	Beta
γ	Gamma
ºC	degree Celsius
M	Molar
mM	Millimolar
µM	Micromolar
g	Gram
mg	Milligram
µg	Microgram
mL	Millilitre
µL	Microlitre
mm	Millimeter
nm	Nanometer
m^2	Meter square
cm^2	Centimetre square
C_t	Cycle threshold
V	Volt
A	Ampere
kDa	kiloDalton
Ω	Ohm
×	Times
× g	Times gravity
±	Plus and/or minus

%	Percent
p	Probability
≤	Less than equal
*	Asterisk
#	Number sign

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In the constitution of selective permeable barrier, the endothelial cells lining inner vascular wall are interconnected with one another by well organized junctional protein complexes which could be categorized into tight junctions (TJs), adherens junctions (AJs), and gap junctions (GJs) (Komarova, Kruse, Mehta and Malik, 2017). Of these categories, the roles of TJs and AJs in governing optimal endothelial permeability are primary and most extensively demonstrated (Tietz and Engelhardt, 2015; Tornavaca et al., 2015). The members of TJs include transmembrane proteins, namely occludin and claudin, as well as cytoplasmic scaffolding protein, namely zonula occluden (ZO) (Vermette et al., 2018). On the other hand, AJs are composed of VE-cadherin and its associated adhesion molecules, namely p120, α-catenin, and β-catenin (Garrett, Lowery, Adam, Kowalczyk and Vincent, 2017). These junctional proteins are highly intermingled with F-actin cytoskeleton in order to maintain endothelial barrier integrity for the regulation of tissue-fluid homeostasis, particularly endothelial permeability. In specific, ZO is located between F-actin cytoskeleton and occludin or claudin, whereas VE-cadherin is linked to F-actin cytoskeleton through α-catenin and β-catenin (Komarova et al., 2017). Under normal physiological condition, the endothelial permeability is preserved at the basal level and gradually increased in response to inflammation to allow the migration of immune cells to the sites of injury (Claesson-Welsh, Dejana and McDonald, 2020). As such, a balanced and optimal homeostasis is deemed to be tightly influenced and regulated by various extracellular proinflammatory stimuli.

Lipopolysaccharide (LPS), also known as endotoxin, is a pathogen-associated molecular pattern (PAMP) found in the outer membrane of Gram-negative bacteria (Sullivan, Bulman and Salamat, 2011). When it is engaged locally or systemically in high concentration, it actively binds to host's pathogen recognition receptor, namely Toll-like receptor-4 (TLR-4) (Li et al., 2017), and triggers a series of proinflammatory signaling pathways including guanine nucleotide exchange factor-H1/Ras homolog family member A/Rho-associated kinase (GEF-H1/RhoA/ROCK) (Guo et al., 2012; Zhou, Guo, Dou, Tang and Huan, 2013), nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) (Cho, Kim, Kim, Ha and Ahn, 2014; Guo et al., 2012; Ma et al., 2004), and mitogen-activated protein kinases (MAPKs) (Cho et al., 2014; Jiang et al., 2013; Kevil, Oshima, Alexander, Coe and Alexander, 2000; Qin, Huang, Mo, Chen and Wu, 2015; Xia, Wang, Wu and Huang, 2014; Yan et al., 2002) pathways, which then results in impaired vascular integrity and endothelial hyperpermeability as a consequence of compromised junctional protein complexes. Remarkably, the activation of proinflammatory signaling pathways could also provoke the overproduction of proinflammatory mediators including various cytokines and chemokines, which further exacerbates interstitial fluid leakage in an unrestricted

manner (Dolmatova, Wang, Mandavilli and Griendling, 2021). These overwhelming vascular leakage and inflammatory responses will lead to endothelial dysfunction, multiple organ failure, and septic shock, ultimately causing death (Hu et al., 2015).

Phloroglucinol is a class of compound accountable for the promising barrier protective effects in human endothelial cells (Bae, 2012). The compound of interest in the present study, 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), is a bioactive compound containing phloroglucinol structural core with hydrophilic acyl group and hydrophobic geranyl group (Ng et al., 2014). It was naturally found in the young leaves of *Melicope pteleifolia* (Champ. ex Benth.) T.G.Hartley, a medicinal plant vernacularly known as 'tenggek burung' (Karim, Nasouddin, Othman, Mohd Adzahan and Hussin, 2011). Preliminary study discovered that tHGA possessed anti-inflammatory activities, as evidenced by its significant inhibitory effects against lipoxygenase (LOX) and cyclooxygenase (COX) enzymes, which are both essential in producing various inflammatory mediators such as cysteinyl leukotriens (CysLTs) and prostaglandins (PGs) (Chong et al., 2016; Shaari et al., 2011). As such, this compound has been extensively studied by employing various experimental models to further explore its protective effects against various inflammatory diseases (Chan, Musa et al., 2021; Chong et al., 2016; Sim et al., 2018) and allergic inflammatory disorders (Ismail et al., 2012, 2019; Lee, Shaari et al., 2017; Lee, Yap et al., 2017; Tan, Israf, Harith et al., 2017; Tan, Israf, Md Hashim et al., 2017; Yap et al. 2018). Remarkably, the study of Chong et al. (2016) demonstrated that tHGA was able to protect endothelial cells against LPS induction via the suppression of vascular inflammation and endothelial hyperpermeability. Interestingly, the inhibition of vascular inflammation was resulted from the attenuation of monocyte adhesion and transmigration across the activated endothelial barrier, through the reduction of cell adhesion molecules (CAMs) and prostaglandin E_2 (PGE_2) production, but not the suppression of monocyte chemoattractant protein (MCP)-1 expression (Chong et al., 2016). On top of that, the same study also elucidated the ability of tHGA to alleviate endothelial hyperpermeability via the inhibition of F-actin cytoskeletal rearrangement (Chong et al., 2016). The findings from this study collectively indicated that the mode of action of tHGA might be due to its direct action on endothelium's structural changes instead of the regulation of proinflammatory mediator secretion.

1.2 Problem Statements

To date, glucocorticosteroids such as dexamethasone are undoubtedly the mainstay for the remedy of inflammatory diseases. Although they are widely used and clinically prescribed, glucocorticosteroids bring numerous adverse drug reactions and severe side effects including liver damage, osteoporosis, and diabetes to the patients (Beckett and Howarth, 2003). Notably, nonsteroidal anti-inflammatory drugs (NSAIDs) and natural product-derived compounds not only have therapeutic values in the treatment of disorders related to endothelial hyperpermeability and vascular inflammation (Liew et al., 2020), but also pose little or no side effect to the patients (Pereira-Leite, Nunes, Jamal, Cuccovia and

Reis, 2017). Therefore, a search for natural compound as potential therapeutic approach for the treatment of inflammatory disorders is urgently needed.

Besides that, previous study has proven that the ability of tHGA to suppress F-actin cytoskeletal rearrangement partly contributed to the abrogation of endothelial hyperpermeability during LPS induction (Chong et al., 2016). As F-actin cytoskeleton is connected to TJs and AJs, and they play collaborative roles in preventing endothelial hyperpermeability which serves as a crucial event in the pathogenesis of various inflammatory diseases including endotoxemia and sepsis (Claesson-Welsh et al., 2020), therefore it is probable that tHGA might possess protective effects on junctional proteins as well. However, the mechanisms of action of tHGA on these junctional proteins are still undefined. As such, in-depth investigations are required to further explore the effects of tHGA on junctional proteins and dissect the signaling pathways mediated by tHGA in suppressing LPS-induced endothelial hyperpermeability.

1.3 Research Objectives

1.3.1 General Objective

To investigate the *in vitro* protective effects of tHGA on cell-cell junctional protein disruption and the underlying signaling pathways in LPS-induced human endothelial cells.

1.3.2 Specific Objectives

1. To examine the effect of tHGA on endothelial junctional integrity in LPS-induced HUVECs via transendothelial electrical resistance (TEER) assay.

2. To assess the effect of tHGA on localization of junctional proteins at cell periphery of LPS-induced HUVECs via immunofluorescence staining.

3. To evaluate the effect of tHGA on junctional protein expression at protein level in LPS-induced HUVECs via Western Blot.

4. To elucidate the effect of tHGA on gene expression of junctional proteins in LPS-induced HUVECs via Reverse Transciption-quantitative Polymerase Chain Reaction (RT-qPCR).

5. To dissect the proinflammatory signaling pathways underlying the junctional protective effects of tHGA in LPS-induced HUVECs via Western Blot.

1.4 Hypotheses

It is hypothesized that tHGA will be able to protect LPS-induced HUVECs by regulating endothelial junctional integrity, junctional protein localization, and the expression of junctional proteins at both protein and gene level, via the inhibition of the activation of various proinflammatory signaling pathways involved in LPS-induced endothelial hyperpermeability.

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