THE EFFECT OF PROPOLIS EXTRACT FROM *Trigona* spp. ON VASCULAR INFLAMMATORY MEDIATORS AND ITS MOLECULAR MECHANISM IN TNF-ALPHA-STIMULATED ENDOTHELIAL CELLS

by

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LIST OF ABBREVIATIONS

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACN	Acetonitrile
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATCC	American Type Cell Culture
BSA	Bovine serum albumin
CAM	Cellular adhesion molecule
CAPE	Caffeic acid phenethyl ester
DMEM	Dulbecco's Modified Eagle' Medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EEP	Ethanolic extract of propolis
E-selectin	Endothelial selectin
FACS	Fluorescence-activated cell sorting
$FeCl_3.6H_2O$	Ferric chloride hexahydrate
$FeSO_4.7H_2O$	Ferrous sulfate heptahydrate
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC	High performance liquid chromatography
HUVEC	Human Umbilical Vein Endothelial Cell
IC_{50}	Half Minimal inhibitory concentration
ICAM-1	Intercellular cell-adhesion molecule-1
Ig	Immunoglobulin
IL-1β	Interleukin-1β
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEP	Methanolic extract of propolis
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECAM	Platelet-endothelial cell-adhesion molecule-1
PGE_2	Prostaglandin E_2
PVDF	Polyvinylidine
Rf	Retention factor
RIPA	Radioimmunoprecipitation assay
ROS	Relative oxygen species
RT-qPCR	Reverse transcription-quantitative real time polymerase chain reaction
SD	Standard deviation

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	Standard error of mean
TBS	Tris buffered saline
TEAC	Trolox equivalent antioxidant capacity
TEMED	Tetramethylethylenediamine
TFC	Total flavonoid content
TLC	Thin layer chromatgraphy
TNF-α	Tumor necrosis factor-α
TPC	Total phenolic content
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
VCAM-1	Vascular cell-adhesion molecule-1
VLA-4	Very late antigen-4
WEP	Water extract of propolis

LIST OF SYMBOLS

%	Percentage
µg	Micro gram
µL	Micro Litre
µM	Micro Molar
mL	Milli Litre
mL/min	Milli Litre per minutes
µL/min	Micro Litre per minutes
nM	nano Molar
^{o}C	Degree celcius
v/v	volume per volume
κ	kappa
α	alpha
β	beta

KESAN EKSTRAK PROPOLIS DARIPADA SPESIS *Trigona* KE ATAS PENGANTARA KERADANGAN VASKULAR DAN MEKANISME MOLEKUL DALAM SEL ENDOTELIAL TERSTIMULASI OLEH TNF-ALPHA

ABSTRAK

Propolis adalah salah satu produk lebah yang mempunyai pelbagai bahan kimia termasuk polifenol, lilin, steroid dan terpenoid. Kandungannya mungkin dipengaruhi oleh sumber tumbuhan, kawasan geografi serta spesies lebah. Pelbagai produk semulajadi telah digunakan sebagai agen anti-radang dan penyembuhan, dan propolis menjadi pilihan yang unik. Oleh itu, objektif kajian ini adalah untuk mengkaji sifat fitokimia dari ekstrak propolis yang terpilih dan kesannya terhadap penghasilan molekul pelekatan, serta mekanisme molekul yang terlibat, terutamanya penyiasatan pada isyarat nuclear factor-kappa B (NF-κB) dalam kultur sel endotelial. Dalam kajian ini, pemeriksaan fitokima fitokima ethanolic extract of propolis (EEP) daripada spesies lebah kelulut tempatan iaitu *Tetrigona apicalis, Geniotrigona thoracica* dan *Heterotrigona itama* diselidiki menggunakan analisis high performance liquid chromatography (HPLC) dan gas chromatography-mass spectrometry (GC-MS). Kandungan fenol dan flavonoid serta aktiviti antioksida termasuk ujian 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) telah dikaji. Kemudian, analisis ‘flow cytometry’ dijalankan untuk mengenal pasti potensi EEP dalam menghalang pengeluaran intercellular adhesion molecule-1 (ICAM-1) dan vascular cell adhesion molecule-1 (VCAM-1) pada human umbilical vein endothelial cell

(HUVEC) yang dirangsang oleh tumor necrosis factor-alpha (TNF-α). Mekanisme molekul pada tahap protin dan gen oleh EEP daripada *G. thoracica* pada isyarat NF-κB telah diperiksa menggunakan ujian 'western immunoblot'. Di samping itu, kajian penghasilan gen NF-κB1 pada tahap mRNA dan molekul pelekatan juga dilakukan oleh analisis 2 langkah reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR). Penyaringan fitokimia menggunakan HPLC mempamerkan ciri kromatografi setiap EEP daripada 3 spesis, tidak sama dengan standard rujukan. Terpenoid adalah salah satu bahan meruwap, merupakan antara sebatian utama yang terdapat dalam semua EEP dalam analisis GC-MS. Di samping itu, 2-methoxy-4-vinylphenol (2M4VP) adalah sebatian utama yang hanya terdapat dalam EEP daripada *G. thoracica*. Korelasi yang kuat telah diperhatikan antara jumlah kandungan fenol dan flavonoid dan sifat antioksidannya (dalam julat antara $R^2 = 0.763$ dan $R^2 = 0.971$); serta penghasilan ICAM-1 (dalam julat antara $R^2 = 0.730$ dan $R^2 = 0.976$) dan VCAM-1 (dalam julat antara $R^2 = 0.835$ dan $R^2 = 0.983$). Analisis 'western blot' menunjukkan peratus penurunan kawalan penghasilan protein IKK, IκB dan NF-κB p65 masing-masing sebanyak 34.7%, 87.3% dan 38.2% berbanding dengan kawalan yang dirangsang oleh TNF-α. Selain itu, hasil RT-qPCR menunjukkan bahawa propolis mengurangkan kawalan VCAM-1- dan NF-κB1-mRNA ke tahap basal selepas rangsangan TNF-α pada HUVEC. Kesimpulannya, EEP daripada *G. thoracica* mengurangkan penghasilan protin dan gen melalui isyarat NF-κB. Oleh itu, EEP ternyata berpotensi sebagai agen anti-radang dan mempunyai ciri-ciri yang bermanfaat untuk rawatan penyakit vaskular.

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ABSTRACT

Propolis is one of the bee products which contained multiple chemical compounds, namely polyphenols, waxes, steroids and terpenoids. Its contents may be influenced by the plant sources, geographical area as well as the bee species. A wide array of natural products have been used as anti-inflammatory and healing agents, with propolis being a remarkable option. Therefore, the objective of this study is to examine the phytochemical properties of the selected propolis extracts and its effects on the expression of adhesion molecules, as well as the molecular mechanisms involved, particularly the investigations on the nuclear factor-kappa B (NF-κB) signalling pathways in cultured endothelial cell. In the present study, the phytochemical screening of ethanolic extract of propolis (EEP) from local stingless bee species namely *Tetrigona apicalis, Geniotrigona thoracica* and *Heterotrigona itama* were investigated using high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analysis. The total phenolic and flavonoid contents, and antioxidant activities which include 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assay were explored. Subsequently, the flow cytometry analysis was carried out to identify the potency of EEP in inhibiting the production of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on tumor necrosis factor-alpha (TNF-α)

stimulated human umbilical vein endothelial cell (HUVEC). The molecular mechanism at protein and gene level of EEP from *G. thoracica* effects on NF-κB signalling pathway was examined using western immunoblot assay. In addition, the gene expression study of mRNA level of NF-κB1 and adhesion molecules was also performed using the 2 steps Reverse Transcription-quantitative Real Time PCR (RT-qPCR) analysis. The phytochemical screening by HPLC exhibited a chromatographic characteristic of each EEP from the 3 species but not similar to reference standards. Terpenoids were the major compounds among the volatile substances found in all EEP of GC-MS analysis. In addition, the 2-methoxy-4-vinylphenol (2M4VP) was the major compound found only in EEP from *G. thoracica*. A strong correlation was observed between the total phenolic and flavonoid content, and its antioxidant properties (range between $R^2 = 0.763$ and $R^2 = 0.971$); and the production of ICAM-1 (range between $R^2 = 0.730$ and $R^2 = 0.976$) and VCAM-1 (range between $R^2 = 0.835$ and $R^2 = 0.983$). The western blot analysis shows the percentage of EEP from *G. thoracica* inhibited the IKK, IκB and NF-κB p65 protein expression about 34.7%, 87.3% and 38.2%, respectively as compared to the TNF-α-stimulated control. Moreover, the RT-qPCR results demonstrated that the EEP from *G. thoracica* reduced the expression of VCAM-1- and NF-κB1-mRNA to the basal levels after the stimulation of TNF-α on HUVEC. In conclusion, EEP from *G. thoracica* is believed to down-regulate the protein and gene expression through NF-κB signalling pathway. Therefore, EEP is revealed to be a potential anti-inflammatory agent and have beneficial characteristics for the treatment of vascular diseases.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The pollination of plants by insects has evolved along with the insects themselves, so the plants and insects are mutually dependent. Bees are the major pollinators in many ecosystems that contain flowering plants (Danforth, 2007). Apart from honey-production and pollination, bees generally produce beeswax, royal jelly, pollen, and propolis from their hives (Kocot *et al.,* 2018). Propolis is a resinous natural product derived from bees, and has been utilized in folk medication in many countries since ancient times (Alday *et al.,* 2016). Stingless bees (*Apidae* family) do not have stings as the stinger is vestigial (Shackleton *et al.,* 2015). Thus, they protect their medically-important food resources by covering the larger holes of the hives with a wax-like substance, and sealing the minute pores of the hives using a special type of resinous substance. The mixture of bee saliva and natural plant resins is called propolis (Kothai and Jayanthi, 2014). Propolis and its extracts have long been used for the prevention and treatment of a variety of diseases. Propolis is used to prevent and treat wounds and ulcers, rheumatism, sprains, heart disease and diabetes, owing to its diverse biological properties (Huang *et al*., 2014). Examples of the properties include antioxidant (Mello and Hubinger, 2012), anticancer (Choudhari *et al.,* 2013), antimicrobial (Kothai and Jayanthi, 2014) and anti-inflammatory (Wang *et al.,* 2013) properties. The usage of propolis as traditional medicines is becoming popular due to the beneficial properties of the propolis constituents. It constituents comprised of polyphenols (flavonoids,

phenolic acids, and their esters), waxes, steroids, terpenoids, vitamins, proteins, amino acids, and sugars (Ahn *et al*,. 2009; Bonvehi and Gutiérrez, 2011; Huang *et al.,* 2014; Russo *et al.,* 2002). The concentrations of these contents depend on the plants nearby the bee hive, climatic conditions when the resin collected by bees (Bankova *et al*., 2000), botanical origins, and bee species (Huang *et al*., 2014). Thus, the chemical composition of propolis varies depending on the source of collection. Several analytical methods have recently been used to identify the chemical compounds, like liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Phenolic plant compounds have gained considerable attention for being one of the main sources of antioxidant activity. Numerous studies have shown that phenolic and flavonoid compounds contributed to the antioxidant activities of natural compounds (Kurek-Gorecka *et al.,* 2014; Silva-Carvalho *et al.,* 2015). Natural compound-based antioxidant substances have a role in preventing the free radical activity (Hernandez *et al.,* 2018; Lobo *et al.,* 2010). Thus, studies on antioxidant substances are currently becoming popular owing to the knowledge of the effects of free radicals on organisms, among others (Aiyegoro and Okoh, 2009). In addition, the immense interest in phytochemical polyphenols like flavonoids, proanthocyanins, and phenolic acids in propolis is in light of the discovery of the active compounds in propolis that are designated as benchmarks in food technology researches. Hence, natural antioxidants are one of the valuable therapeutic agents that can reduce the occurrence of illnesses triggered by oxidative stress (Lobo *et al.,* 2010). Besides that, the flavonoids and phenolic compounds present a crucial function as anti-inflammatory agents (Arulselvan *et al*., 2016). Flavonoids inhibit the endothelial induction of cell-adhesion molecules in TNF-α-activated human endothelial cells,

apart from protecting against oxidant-induced endothelial cell damage (Crespo *et al*., 2008). Endothelial cells (ECs) activated once getting the response from pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS) causing the production of pro-inflammatory cytokines namely Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), apart from recruiting leukocytes (Mai *et al.,* 2013). They selectively express surface adhesion molecules like intercellular cell-adhesion molecule-1 (ICAM-1), vascular cell-adhesion molecule-1 (VCAM-1) and endothelial selectin (E-selectin) that control the process of leukocyte-endothelial interactions (Mai *et al.,* 2013). Under inflammatory conditions, cytokines such as IL-1β or TNF-α activate the EC, thus increase the adhesion molecules expression and the presentation of chemokines on the cell surfaces. These substances then participate in the neutrophil adhesion-recruitment cascade (Crespo *et al*., 2008). Cytokines are released into the bloodstream following the onset of many different pathophysiological stimuli. Furthermore, the activated EC contribute secondarily to inflammation-mediated tissue destruction *via* the release of cytokines (Kim *et al*., 2012). These factors result in acute or chronic inflammatory responses and have been implicated in the pathogenesis of many diseases (Wang *et al*., 2013). Therefore, the inhibition of cytokine production or function is a key mechanism in the control of inflammation (Kim et al., 2012). There is an increasing evidences proving that the transcription factor of nuclear factor kappa B (NF-κB) as well as the activator protein 1 (AP-1) play vital functions in inflammatory responses by regulating the transcriptional activation of many inflammation-related genes (Wang *et al*., 2013). NF-κB serves as a central mediator of the pro-inflammatory gene that responds to a large variety of immunological receptors (Liu *et al*., 2017). Since the deregulation of NF-κB activation is involved in the pathogenesis of various chronic inflammatory diseases like chronic obstructive

pulmonary disease, rheumatoid arthritis, atherosclerosis, and inflammatory bowel disease (Damen *et al*., 2017), aiming the NF-κB signalling pathway is an attractive approach in anti-inflammatory remedies. Therefore, an understanding of the underlying mechanisms of the pathological activation of NF-κB in individual diseases is crucial in designing more specific and effective therapeutic agents for the treatment of inflammatory diseases. In general, flavonoids, which are the important constituents of propolis, are possible healing agents for chronic inflammatory diseases (Ginwala *et al.,* 2019) since these agents suppress vascular inflammation presumably through manipulations of the NF-κB signalling pathway (Lim *et al.,* 2019; Liu *et al.,* 2017). Therefore, investigations on the effectiveness of therapeutic strategies against chronic inflammation, particularly those which target the underlying vascular inflammation, are urgently needed to overcome the global health burden of unresolved and increasingly prevalent chronic diseases.

1.2 Problem statement

It is known that honey, pollen, and propolis are rich in phenolic compounds which act as natural antioxidants, and that these stingless bee products are becoming increasingly popular because of their potential uses in the enhancement of human health. For the past couple of years, the emergence of anecdotal claims pertaining to the medicinal values of stingless bee products, including propolis, has been overwhelming. However, lack of evidence is available from the scientific research's point of view, especially on the underlying molecular mechanisms of propolis-induced antioxidant and anti-inflammatory effects of propolis derived from Malaysian stingless bees on EC.

1.3 Objectives

To examine the phytochemical properties of the selected propolis extracts and its effects on the expression of adhesion molecules, as well as the molecular mechanisms involved, particularly the investigations on the NF-κB signalling pathways in cultured endothelial cells.

The specific aims of this study are as follows:

1. To study the phytochemical profile, total phenolic and flavonoid contents of different propolis extracts namely *Tetrigona apicalis, Heterotrigona itama* and *Geniotrigona thoracica*,
2. To evaluate their antioxidant activities and correlate between the aforementioned classes of compounds.
3. To identify the most potent propolis extract that could inhibit the upregulation of TNF-α-induced ICAM-1 and VCAM-1 in HUVEC EA.hy926.
4. To elucidate the molecular mechanisms by which the selected propolis extract down-regulated the expression of above mentioned proteins through investigations on NF-κB pathways.
5. To examine the effects of selected propolis extract at the mRNA level of NF-κB1, ICAM-1 and VCAM-1 expression *via* RT-qPCR analysis.

1.4 Flow chart of study design

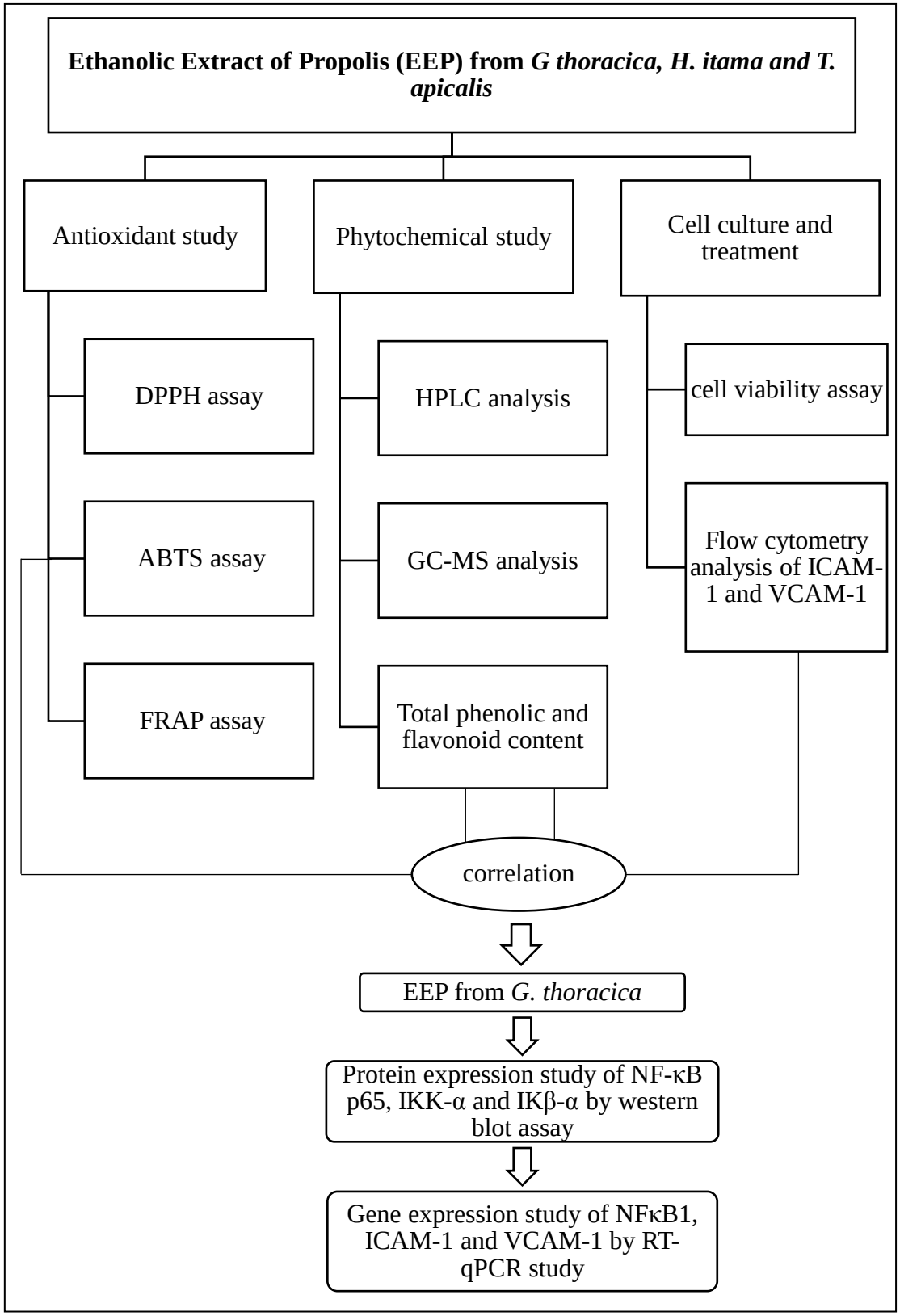

Figure 1.1 : The study design for the molecular mechanism of EEP from Malaysian stingless bees through NF-κB pathway.

1.5 Hypotheses

1. Propolis predominantly contains flavonoids and phenolic acids that contribute greatly to its antioxidant and anti-inflammatory activities.

2. As a potent antioxidant and anti-inflammatory agent, propolis down-regulate the activation of the nuclear factor-kappa B (NF-κB) pathway, thus inhibiting the expression of a few pro-inflammatory mediators or cytokines, namely endothelial adhesion molecules (ICAM-1 and VCAM-1) and tumor necrosis factor-α.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of propolis

2.1.1 Malaysian stingless bees

Both stingless bees (*Meliponini*) and honeybees (*Apis*) are vital pollinators of crops in Southeast Asia (Inoue, 1985). They are the major visitors of many flowering plants in tropical and many subtropical parts of the world (Heard, 1999). Unlike honeybees, stingless bees are less harmful to humans and domesticated animals which characteristically do not sting in defence (Hasan *et al.,* 2014). In addition, the quantity of propolis from stingless bee is greater than the other bees (Hasan *et al.,* 2014; Ibrahim *et al.,* 2016). Generally, stingless bees belonging to the Apidae family are small to medium in sized which have vestigial stings (Heard, 1999). They mate only once, do not use water to cool their nests or pure wax to build it, and cannot freely swarm to reproduce but instead must first make a new hive. The males feed on flowers, since the gravid queens cannot fly (Roubik, 2006).

Stingless bees are mainly found in primary rainforests and continuously-disturbed areas which have various flowering plants (Inoue, 1985). The estimated several hundred species of stingless bees are grouped into 21 genera. The ranks of the groups have varied recently as these groups have been designated as tribes. *Melipona* and

Trigona are the most vital genera of stingless bees. Studies have shown that *Melipona* consisted of more than 50 species (Heard, 1999). Meanwhile, *Trigona* is the largest and most widely-distributed genus, with more than 130 species in approximately 10 subgenera - including the neotropical *Trigona sensu stricto* and most of the Asian *Meliponini* (Heard, 1999). Kothai and Jayanthi (2014) stated that *Trigona* spp. are exclusive to tropical and sub-tropical areas. In Malaysia, 17 to 32 species of stingless bees have been identified such as *Trigona (Heterotrigona) itama, Trigona (Geniotrigona) thoracica* (Kelly, 2014), and *Trigona (Tetrigona) apicalis* are mostly used in meliponiculture.

Stingless bees are basically named according to their physical characteristics and the shapes of their hives. Cresson (1878) stated that *G. thoracica* is about 0.22 inches in length, with a short golden-sericeous abdomen and yellowish sub-hyaline wings. *H. itama*, which is most abundant in secondary forests, has a medium-sized body (6.15 mm). In general, the colony size of *H. itama* is moderately large and the guard bees are more aggressive at the nest (Inoue, 1985). This species is widely distributed throughout the Malayan Region (Siam, Malaya, Sumatra, Bangka Is., Borneo and Java) (Sakagami, 1959). **Figure 2.1** shows different types of nest entrance of stingless bee species. The nest entrance is varied depending on the genus. The external characteristics of nest and entrance tubes of several Malaysian stingless bees shown in **Table 2.1** and **Table 2.2**, respectively (Kelly *et al.*, 2014). Overall, *T. thoracica* have bigger size of nest and entrance tube compare to the other species. In addition, the shape of the entrance tube is mount and hard as well as black and brown in colour.

Figure 2.1 : The shape of the nest entrance of stingless bees from *G. thoracica* (A), *T. apicalis* (B), and *H. itama* (C).

Table 2.1 : External characteristics of nests of stingless bee species (Kelly *et al*., 2014).

Species	Mean ± s.d (cm)			
	Height of three trunk	Circumference of the three trunk (top)	Circumference of the three trunk (bottom)	Height of entrance from bottom
T. thoracica	108.7 ± 43.7	105.3 ± 23.7	111.6 ± 17.5	54.7 ± 29.8
T. itama	86.2 ± 20.2	96.3 ± 24.9	100.4 ± 27.1	45.8 ± 21.1
T. terminate	102.3 ± 22.1	108.5 ± 34.4	125 ± 39.3	44.8 ± 34.6
H. scintillans	45	69	110	45
T. laeviceps	70.3 ± 42.2	84.3 ± 1.52	101.7 ± 18.9	37.7 ± 37.8

Table 2.2 : Size and descriptions of entrance tubes of stingless bee species (Kelly *et al*., 2014).

Species	Entrance tube (mean ± s.d) (cm)			Shape	Colour	Rigidity
	Length	Width	Tube length			
T. itama	1.53 ± 0.47	2.04 ± 0.69	7.84 ± 7.39	F, R	BR, Lb	S, H
T. thoracica	3.97 ± 1.29	4 ± 0.92	7.38 ± 3.65	M	Br. Bl	H
T. terminate	1.84 ± 0.38	1.96 ± 0.1	7 ± 2.02	F	Lb	S
T. laeviceps	1.85 ± 0.35	2.75 ± 0.75	4.25 ± 1.75	F	Bl	S, H
H. scintillans	NA	NA	NA	R	Br	S

Entrance shape: F=Funnel, M=Mount, R=Round-ringed; Colour: Br=Brown, Bl=Black, Lb=Light Brown; Rigidity: S=soft, H=hard; *NA: Not available; entrance was accidentally destroyed

2.1.2 Botanical characteristics of propolis

Propolis is a resinous natural substance collected by bees from the buds, leaves, and exudates of various trees as well as plants (Sanpa *et al.*, 2015). It contains a combination of natural resins, wax, and bee enzymes (Mello and Hubinger, 2012). Propolis, in Greek, means defending of a city or beehive. Thus, the bees are involved in the defence of their communities against enemies like bacteria and other insects (Hasan *et al*., 2014). The waxy nature of propolis is exploited by the bees to seal the openings and smoothen the internal walls of the nest, apart from maintaining the temperature as well as keeping out wind and rain (Kothai and Jayanthi, 2014). Propolis is sticky beyond 25ºC; it turns out to be hard and brittle at lower temperatures. It will become more sticky and gummy over 45°C and turn into liquid at high temperatures (60°C to 70°C) (Wagh, 2013). In addition, the type of propolis may vary according to parameters like smell, colour, constitution, and chemical composition, all of which depend mainly on the floral area from which it is collected (Bonvehi and Gutiérrez, 2011) as well as the species of the bee (Cottica *et al*., 2011).

2.1.3 Chemical constituents of propolis

Over 150-180 active compounds have been identified in propolis (Hasan *et al*., 2014, Mello and Hubinger, 2012). These include polyphenols (phenolic acids, flavonoids, and their esters), terpenoids, steroids, beeswax, bioelements, as well as other components like vitamins, proteins, amino acids, and sugars (Ahn *et al*., 2009; Bonvehi, 2011; Russo *et al*., 2002). Further examples of compounds include eugenol, anethole, hydroquinone, pterostilbene, and naphthalene (Kurek-Gorecka *et al*., 2014). The chemical compositions of propolis have been extensively studied in many

countries such as Brazilian, Europe and Canary Islands (Ibrahim *et al.,* 2010). Numerous studies have shown that the composition of propolis is depending on the plants at the area of collection, as well as the region of origin (Bankova *et al.,* 2000; Socha *et al.,* 2015). In addition, Kothai and Jayanthi (2014) stated that the characteristics were influenced by geographical factors, vegetation, and season, whereby these factors indirectly diversified the biomedical applications.

Researchers have shown interest in the investigation of isolated compounds that are responsible for the actions of propolis. Tests for phytochemical screening by TLC in methanolic extract of propolis (MEP) from Malaysian *H. itama* revealed the presence of terpenoids, flavonoids, phenols, essential oils, steroids, saponin and coumarins (Ibrahim *et al.,* 2016). In addition, Nazir *et al.* (2018) studied on the ethanolic extract of propolis (EEP) from Malaysian *G. thoracica* by GC-MS analysis. The main identified phenolic compound and terpenoid were 1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl) and fren-9(11)-en-2.alpha.-ol, respectively. Other than that, the study of EEP from Brazilan stingless bees *Tetragonisca fiebrigi* determined by GC-MS analysis found phenolic compounds, alcohol, and terpenes as its major class compounds (Sanpa *et al.,* 2015). Additionally, Sawa *et al*. (2007) isolated six xanthones, one triterpene and one lignane from the EEP of *Tetragonula laeviceps* from Thailand by using GC-MS analysis.

2.1.4 Historical and current uses of propolis

The usage of propolis as a medical remedy has a long history in many countries, dating back at least to 300 BC (Yang *et al*., 2011). Antioxidants are well-known to show a vital role in reducing the risk of chronic diseases like carcinogenesis, diabetes, and atherosclerosis (Yang *et al*., 2011). Many studies have reported that propolis contains a variety of active compounds and represents a wide range of biological effects, which makes it as a good antioxidant additive that increases its potential in traditional medicine (Aliyazicioglu *et al*., 2013). Over the recent years, natural products have become a subject of immense interest for food producers and consumers. Among them, bee products especially propolis are worthy of greater attention owing to their health-promoting and therapeutic properties (Socha *et al.*, 2015). Propolis has gained popularity for its ability to be used as health drinks and foods as well as medicine and cosmetics (Cauich-Kumul and Campos, 2019; Wagh, 2013). Beverage additives containing propolis extracts can be found in the market in numerous countries (Mello and Hubinger, 2012; Trusheva *et al*., 2007). The hard, resinous nature of propolis which is a product that cannot be consumed in its natural form, has generated interest among scientists to develop a way to prepare propolis solutions. For example, it can be extracted from an alcoholic or aqueous medium and transformed into powder (Mello and Hubinger, 2012).

2.1.5 Pharmacological activity of propolis

Propolis is well known for its pharmacological activities due to the diversity of its chemical compositions such as anticancer, antioxidant, antibacterial, anti-microbial, anti-inflammatory and many more. The anticancer property of EEP was reported by Choudhari *et al.* (2013) testing the cytotoxic and apoptotic effects in four different cancer cell lines namely MCF-7, HT-29, CaCo-2 and B16F1 at different concentration. The EEP revealed a concentration and time dependent cytotoxic effect. Additionally, Ibrahim *et al.* (2016) studied the antibacterial activities of MEP from Malaysian stingless bees namely *H. itama* and *G. thoracica*. Both extract inhibited the growth of *Staphylococcus aureus*. The MIC value shows that the most effective propolis was *H. itama* (5 mg/mL).

Moreover, the EEP from *Tetragonisca fiebragi* and *Melipona arbignyi* found in Brazil was active against all types of microorganism tested and showed antioxidant activity by scavenging free radicals, inhibiting haemolysis and lipid peroxidation in human erythrocytes incubated with an oxidizing agent (Campos *et al.,* 2014; Campos *et al.,* 2015). Furthermore, the EEP from *Scaptotrigona* spp. shows antioxidant activity using DPPH method (Sawaya *et al.,* 2009). In addition, the 70% EEP produced by *Trigona* spp. from Indonesia also displayed strong antioxidant activity against DPPH scavenging method (Hasan *et al.,* 2014).

Propolis is also notable for its anti-inflammatory properties. Study by Guzman-Gutierrez *et al.* (2018) revealed the anti-inflammatory activity of ethyl acetate propolis extract from Mexican stingless bee *Melipona beecheii.* In addition, Santos *et al.* (2017) studied on the anti-inflammatory activity of the hydroalcoholic extract from stingless bee *Melipona orbignyi*. The result shows that propolis inhibited the activity of the inflammatory enzyme hyaluronidase. Silva-Carvalho *et al*. (2015) studied the anti-inflammatory mechanisms of propolis that have been investigated worldwide such as Europe, Brazil, Mediterranean, China and Nepal (**Table 2.3**). Most of the isolated compounds found were CAPE and caffeic acid. The overall results of different studies have provided compelling evidence of the anti-inflammatory properties of propolis extracts and its derivatives when tested *in vivo* and *in vitro*. Taken together these results indicated that propolis has therapeutic potential for the treatment of many diseases.

Table 2.3 : Anti-inflammatory mechanisms of propolis (Silva-Carvalho *et al*., 2015).

Origin	Propolis type/ plant source	Type of extract/ isolated compound(s)	Species/cells	Effect
Purchased: Sigma Aldrich Co. synthesized	Characteristic of European, Brazilian, and Mediterranean propolis	Caffeic acid, quercetin, naringenin, CAPE	Peritonal macrophages	Suppression of lipoxygenase pathway of arachidonic acid metabolism
Croatia	European propolis	Water-soluble derivatives, PEE	Swiss albino mice Male Swiss albino mice	Reduction of DNA damage in peripheral lymphocytes Suppression of functional activity of macrophages
Purchased: Sigma Aldrich Co.	Characteristic of European type propolis	CAPE	J774 macrophages, Male Wistar rats	*In vitro* and *in vivo* of cyclooxygenase-1 and -2 activity
Purchased: Wako Pure Chemical Industries, Ltd.			Male Wistar albino rats	Decrease in polymorphonuclear leukocyte infiltration in lung tissues
			Gastric epithelial cell line (AGS), *H. pylori* (strain NCTC 11638)	Inhibition of *H. pylori*-induced NF-κB and AP-1-DNA-binding activity, prevention of IKBα degradation in AGS and suppression of TNF-α
			RAW 264.7 macrophages	Decrease production of I-1β, monocytes chemoattractant protein 1, and the production and expression of TNF-α
			Male swiss inbred strain mice	Decrease of cyclooxygenase 2 expression, NF-κB activity, c-Jun-Nterminal kinase, IKK and IKβ phosphorylation
Brazil Purchased: Acros Organics	Green propolis Characteristic of European, Brazilian, and Mediterranean propolis	PEE Caffeic acid	RAW 264.7 macrophages	Downregulation of NF-κB, p38 MAP kinase, and c-Jun-Nterminal kinase

Table 2.3. Continued.

Brazil	Green propolis	PEE	Sprague-Dawley rats	Inhibition of carrageenan-induced rat hind paws edema and the chemotaxis of human polymorphonuclear leukocytes (PMNs)
Synthesized	Characteristic of European type propolis	CAPE	Polymorphonuclear leukocytes obtained from Human blood	Inhibiton of 5-lipoxygenase activity and arachidonic acid releasse
Chile	European propolis	PEE	Male CF-1 mice	Inhibition of NO release by the macrophages
China	European propolis	PEE and PWE	Male ICR mice and male Wistar rats	Inhibition on the activation and differentiation of mononuclear macrophages
Brazil	Green propolis	PWE	Swiss and BALBC mice	Decreade in the number of macrophages and neutrophils
	Red propolis	PEE	Male Wistar rats	Decrease in renal macrophage infiltration in rats with chronic kidney disease
Nepal	Nepalese propolis	PEE	Bone marrow-derived mass cells (BMMC)	Inhibiting IL-6, TNF-α, and IL-13 gene expression and inhibiting the activation of IKK leading to NF-κB inactivation

2.2 Inflammation

2.2.1 Innate vs adaptive immunity

Inflammation is an essential mechanism to protect the human body from foreign substances. The aim of inflammation is to replace injured tissue and initiate the healing process (Angajala and Radhakrishnan, 2014). Inflammation is the outcome of complex interactions between immune cells, their mediators, as well as regulators; all of which are part of the innate immune response (Gallo *et al*., 2017). The innate or non-specific immune response is the first line of defence against pathogen. It is an immediate response that provides the physical and chemical barriers in order to prevent the spread of pathogens from the host (Sathe *et al.*, 2014). The cells of the innate immunity mainly macrophages, neutrophils, mast cells, basophils, and eosinophils play a crucial parts in initiation and elimination of pathogens that have been targeted by an adaptive immune response (Janeway *et al.*, 2001).

In contrast to adaptive or acquire immunity, the response effects is long-term and highly specific to the pathogen presented as well as has memory. This second line of defence is mediated by the presence of lymphocytes either T or B cells following exposure to a specific antigen (Snyder, 2017). In response to tissue injury, pro-inflammatory cytokines such as Interleukin (IL-1β, IL-6), tumour necrosis factor-α (TNF-α), and mediators (e.g. prostaglandin E_2; PGE_2) play a crucial roles in activating the immune cells (Kim *et al*., 2015). The overproduction of these pro-inflammatory mediators is commonly related to pro-inflammatory stimuli, which triggers acute or chronic inflammatory responses that are involved in the pathogenesis of various

illnesses (Wang *et al.*, 2013). Thus, many complex biochemical processes are needed to trigger the cellular inflammatory reactions (Kim *et al*., 2015).

2.2.2 Acute vs chronic inflammation

Acute inflammation is a short-term process which occurs and subsides in response to tissue damage (Oishi and Manabe, 2016) to form scar tissue (Ramos and Miranda, 2007). The damaged tissue is characterised by five cardinal signs, which are swelling, heat, pain, redness and loss of function (Gallo *et al.,* 2017). The resolution of acute inflammation is an active process wherein anti-inflammatory signals suppress inflammation, clear immune cells and promote healing, which subsequently restore the normal tissue function (Oishi and Manabe, 2016). In contrast, chronic inflammation is the result of long-term damage, whereby continuous injury, inflammation, and repair occur simultaneously. It happens when the acute inflammatory response is not sufficient for the elimination of injuring agent and the restoration of tissues to their normal physiological states (Oishi and Manabe, 2016). Hence, only low concentrations of the chemical mediators are present. The neutrophils, which are usually present in acute inflammation, are substituted by lymphocytes, plasma cells, fibroblasts and macrophages (Prentice, 2011). The damage occurs to the connective tissues as the low-grade inflammation persist results in tissue fibrosis and necrosis, thus extending the curing and repair processes (Oishi and Manabe, 2016; Prentice, 2011).Repeated tissue damage and degeneration may lead to unresolved vascular inflammation (Ramos and Miranda, 2007).

2.3 Vascular inflammation

2.3.1 Role of endothelial cells in vascular inflammation

The recruitment of circulating leukocytes, primary monocytes, macrophages, and T lymphocytes into the vascular wall is the hallmark of vascular inflammation (Brasier, 2010). In particular, the circulatory leukocytes are recruited into vascular tissue through a coordinated process of cellular adhesion, chemotaxis and subsequent leukocyte activation which initiated by non-specific vascular injury. This recruitment process is mainly the consequence of coordinated expression of vascular adhesion molecules, chemotactic functions and cytokines (Brasier, 2010). ECs play a key role in physiological processes such as nutrient delivery, immune cell trafficking, blood supply, and metabolic homeostasis, as well as pathological processes such as inflammation (Danese *et al.,* 2007).

ECs which line the internal surface of the vasculature, are part of a complex system that regulates vasodilation and vasoconstriction, growth of vascular smooth muscle cells, inflammation and haemostasis, maintain a proper blood supply to tissues and regulating inflammation and coagulation (Zhang, 2008). An understanding of the differences between the signalling pathways of inflammatory factors is absolutely necessary. Thus, inflammation control at the level of ECs may efficiently retard or reverse the pathogenetic process (Mako *et al.,* 2010). Human umbilical veins endothelial cells (HUVEC) model are probably the most widely-used source of human ECs since they are useful for any research on general properties of human endothelium and represent the most simple human vascular cell type (Siow, 2012).

2.3.2 Leukocyte-endothelial interactions in inflammation

Leukocyte-endothelial interactions in inflammation are triggered by either exogenous stimuli (eg: temperature, light, magnetic field and ultrasound) or endogenous stimuli (eg: changes in pH, redox, gradient and enzyme constituents) (Hatakayama, 2017). The interactions between the leukocytes and the endothelium involve a variety of adhesive as well as migratory molecular events. These include (1) low-affinity, transient, and reversible rolling adhesions; (2) integrin-dependent firm adhesive interactions; as well as (3) migration of the leukocytes through the endothelium and beyond (Langer and Chavakis, 2009). The penetration of the basement membrane and migration to the interstitial space is known as transendothelial migration.

Leukocytes extravasate from blood to the site of inflammation *via* a 'three-step' paradigm of inflammatory cell recruitment which are involves rolling, activation, and adhesion as shown in **Figure 2.2**. During the rolling process, the contact of leukocytes with the luminal endothelial surface allows the leukocytes to effectively 'detect' the endothelial surface-bound chemokines (Middleton *et al.,* 2002). The chemokines are dramatically induced by inflammatory mediators during the rolling of the leukocytes (Langer and Chavakis, 2009). The chemokines induce intracellular signals by interacting with the G-protein-coupled chemokine receptors on the leukocytes, leading to an inside-out integrin activation (Granger and Senchenkova, 2010).

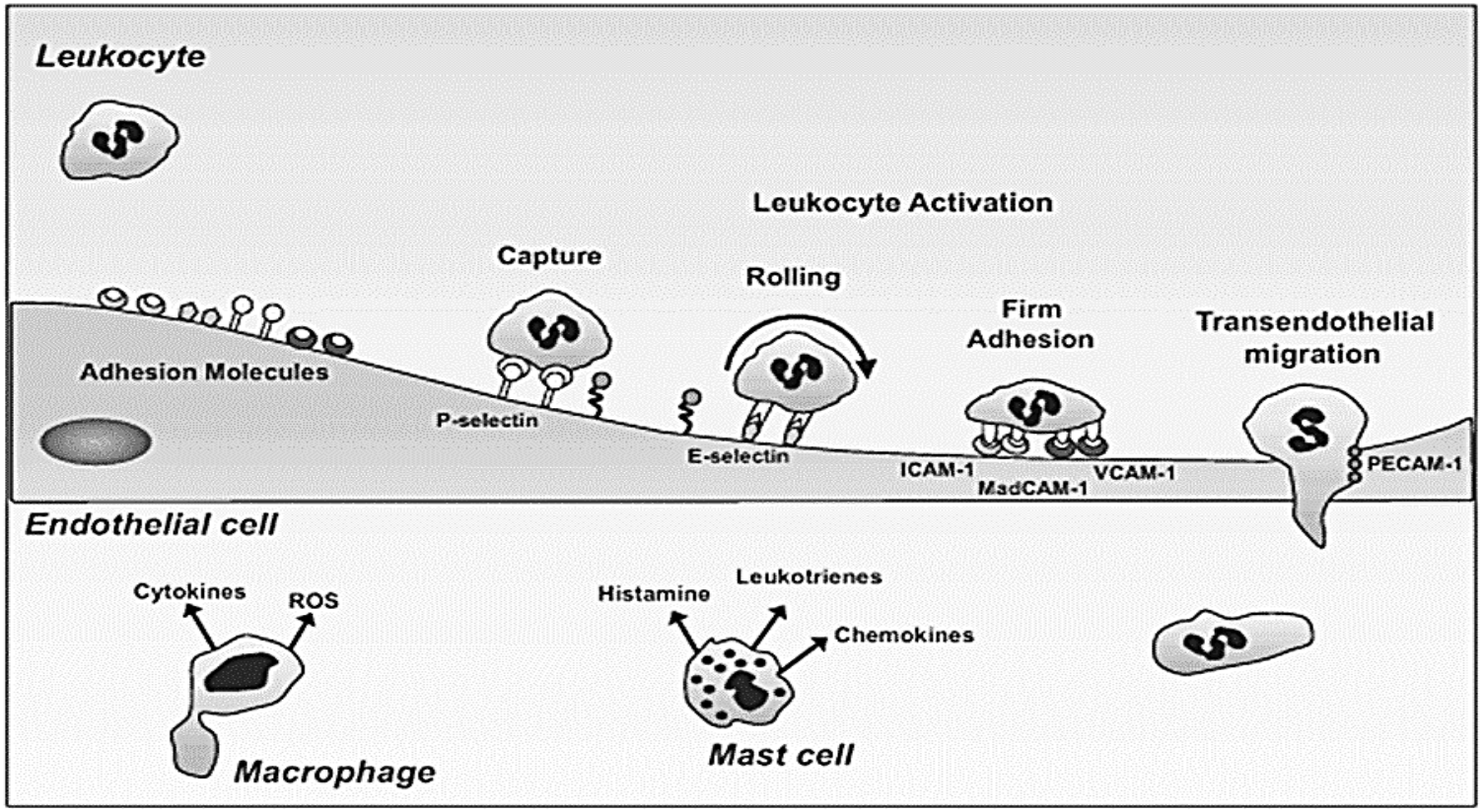

Figure 2.2 : Leukocyte-endothelial adhesion molecules step during inflammation (Granger and Senchenkova, 2010).

The leukocytes adhere firmly to the endothelium, apart from changing their shapes and forming pseudopodia. These shape changes are associated with the conversion of G-actin to F-actin, hence enabling the cells to adhere and later transmigrate (Middleton *et al.,* 2002). ICAM-1 and ICAM-2 are constitutively expressed; ICAM-1 expression is further increased following endothelial activation. In contrast, endothelial VCAM-1 is recognised by the β-1 integrin receptors that are predominantly found on lymphocytes and monocytes. This adhesion pathway appears to be responsible for the immune functions that occur in the absence of β2 integrins in leukocyte adhesion deficiency-1 patients (Langer and Chavakis, 2009). The adhesive activity of integrins is regulated by alterations in integrin affinity and valency (Wehrle-Haller, 2005), whereby the former is mediated by conformational changes of the integrin subunits while the latter involves changes in the distribution of integrin on the cell surface (Langer and Chavakis, 2009).

According to Langer and Chavakis (2009), leukocyte-endothelial adhesion molecules can be grouped into three families. The first group is selectins, which are a family of three carbohydrate-recognising molecules, namely E-selectin and P-selectin which are expressed on the surface of endothelium, and L-selectin expressed on the surface of neutrophils. P-selectin is constitutively stored in distinct EC granules that are rapidly mobilized to the EC surface where P-selectin gets homogeneously distributed on the cells (Schnoor *et al.,* 2015). The second group is integrins, which are heterodimers comprising α- and a β-chain in which it can recognise multiple ligands (Campbell and Humphries, 2011) including complement factors, cell surface glycoproteins, extracellular matrix proteins, as well as soluble components of the haemostatic and fibrinolytic cascades (Langer and Chavakis, 2009). The third group consists of major