IMMUNOREGULATORY EFFECT OF ETHANOLIC EXTRACT PROPOLIS (EEP) FROM *Geniotrigona thoracica* sp. ON THE FORMATION OF THP-1 DERIVED MACROPHAGE FOAM CELLS

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by

MUHAMMAD SYAMIL BIN MOHD SUIB

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I humbly dedicate this thesis to my beloved father and mother En. Mohd Suib Bin Hj. Hashim and Pn. Zuraida Binti Mohamad for their endless love, support and prayers in every step of the way that I take in my life. I would like to thank my sisters, Syazana Binti Mohd Suib and Syazliana Binti Mohd Suib for always be there for me and encourage me in everything I did. Thank you.

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LIST OF ABBREVIATIONS

CAPE	Caffeic Acid Phenetyl Ester
CD	Cluster of Differentiation
CE	Cholesterol ester
COX 1/ COX 2	Cyclooxygenase 1 or 2
CVD	Cardiovascular disease
EC	Endothelial cell
EC_{90}	90% maximal effective concentration
ECM	Extracellular matrix
EEP	Ethanolic extract of propolis
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ICAM-1	Intracellular adhesion molecules-1
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
LDL	Low density lipoprotein
MCP-1	Monocyte chemo attractant protein-1
MCSF	Macrophage-colony-stimulating factor
MMP	Matrix metalloproteinases
NF-kB	Nuclear Factor kappa B
NO	Nitric Oxide
OH	Hydoroxyl radical
ONOO-	Peroxynitrite
ORO	Oil Red O
oxLDL	Oxidised Low Density Lipoprotein
PBS	Phosphate Buffer Saline

PKC	Protein Kinase C
PMA	Phorbol-12-myristate 13 acetate
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCD	Sudden cardiac death
SMC	Smooth muscle cell
TGF-β	Transforming growth factor beta
TIC	Total Ion Chromatogram
TNF-α	Tumor Necrosis Factor alpha
t_R	Retention time
VCAM-1	Vascular adhesion molecules-1
VD3	1,25-dihydroxyvitamin D3
VSMC	Vascular smooth muscle cells

KESAN PENGAWALATURAN IMUN EKSTRAK PROPOLIS DARI *Geniotrigona thoracica* sp. KE ATAS PEMBENTUKAN SEL BUSA MAKROFAJ DIPEROLEH DARIPADA SEL THP-1

ABSTRAK

Propolis adalah bahan resin kompleks yang dikumpulkan oleh lebah dari tunas dan eksudat tumbuhan yang telah dilaporkan untuk menunjukkan pelbagai potensi terapeutik. Penggunaan produk semulajadi seperti propolis telah digunakan untuk rawatan pelbagai penyakit kronik seperti aterosklerosis. Aterosklerosis adalah penyakit radang kronik yang membabitkan pembentukan sel busa makrofag daripada monosit di dalam arteri, yang memainkan peranan penting dalam aterogenesis. Banyak kajian menunjukkan bahawa propolis yang dihasilkan oleh lebah madu boleh merencat pembentukan aterosklerosis tetapi masih tiada kajian yang dilakukan menggunakan propolis yang dihasilkan oleh lebah Indo-Malayan. Oleh itu, kajian ini dijalankan untuk mengkaji kesan propolis daripada spesies *Geniotrigona thoracica* pada pembentukan sel busa yang terlibat dalam atheroscelrosis. Ekstrak etanol propolis (EEP) daripada tiga koloni lebah dari spesies *Geniotrigona thoracica* yang berbeza dikumpulkan dan diekstrak menggunakan etanol 80%. Pelbagai sebatian yang tidak menentu yang terdapat dalam ekstrak propolis dianalisis melalui analisis GC-MS dan HPLC. Kepekatan optimum propolis ekstrak (0-200 μg/ml) untuk merawat makrofaj diperoleh daripada sel THP-1 ditentukan melalui ujian *in vitro* daya kehidupan sel dengan menggunakan reagen PrestoBlue®. Sel THP-1 dibezakan dengan makrofag yang diperoleh daripada sel THP-1 menggunakan 100 ng/ml PMA selama 72 jam. Kemudian, makrofag yang diperoleh daripada sel THP-1 dirawat dengan 80 μg/ml LDL teroksida dan 20 μg/ml EPP selama 6, 24 dan 48 jam. Penampilan morfologi sel

buih ditentukan menerusi pewarnaan oil red O manakala jumlah ester kolesterol yang terdapat di dalam sel-sel buih dianalisis dengan menggunakan Kit Sokongan Kuantifikasi Kolesteril Ester. Akhirnya, tahap rembesan sitokin keradangan iaitu TNF-α dan IL-1β yang dirembes oleh sel-sel yang dirawat diukur oleh ELISA. EEP dari spesis *Geniotrigona thoracica* didapati mengandungi banyak sebatian triterpenoids dan kehadiran asid kafein, asid *p*-Coumaric dan sebatian quercetin berdasarkan analisis GC-MS dan HPLC. Propolis dari koloni 2 dipilih untuk digunakan dalam kajian ini kerana ia mempunyai lebih banyak sebatian bioaktif. EC_{90} EEP ditentukan berada pada kepekatan 20 μg/ml dan kepekatan ini digunakan untuk eksperimen berikutnya. Pemerhatian morfologi menunjukkan bahawa butiran lipid kelihatan berkurang di sitosol sel makrofag yang diperoleh daripada THP-1 yang dirawat propolis dan LDL teroksida. Di samping itu, kandungan ester kolesteril telah berkurangan secara signifikan dalam sel makrofaj yang diperoleh daripada THP-1 yang menerima perawatan propolis dan LDL teroksida pada setiap masa rawatan. Selain itu, EEP juga didapati dapat mengurangkan dengan ketara rembesan sitokin TNF-α dan IL-1β dalam supernantan propolis dan LDL teroksida yang dirawat THP-1 yang diperolehi makrofaj. Sebagai kesimpulan, EEP dari spesis *Geniotrigona thoracica* menghalang pembentukan sel busa makrofag yang berasal dari sel THP-1 dengan menghalang rembesan TNF-α dan IL-1β yang biasanya dirembes secara banyak semasa pembentukan aterosklerosis.

IMMUNOREGULATORY EFFECT OF ETHANOLIC EXTRACT PROPOLIS (EEP) FROM *Geniotrigona thoracica* sp. ON THE FORMATION OF THP-1 DERIVED MACROPHAGE FOAM CELLS

ABSTRACT

Propolis is a complex resinous substances collected by bees from buds and exudates of plants that has been reported to demonstrate various therapeutic potential. The usage of natural product such as propolis has been used for treatment of various chronic diseases which includes atherosclerosis. Atherosclerosis is a chronic inflammatory disease involving the formation of monocyte derived macrophage foam cells in arteries, which play a major role in atherogenesis. Studies showed that propolis produced by honeybees may inhibit atherosclerosis but there is no studies that have been performed using propolis produced by Indo-Malayan stingless bees. Hence, this study was carried out to investigate the effect of propolis from *Geniotrigona thoracica* species on the formation of foam cells. Propolis from three different colonies of *Geniotrigona thoracica* species were extracted using 80% ethanol. The volatile and non-volatile compounds present in ethanolic extract of propolis (EEP) were analysed via GC-MS and HPLC analysis. The optimal concentration of EEP (0-200 µg/ml) to treat THP-1 derived macrophages was determined via *in vitro* cell viability assay by using PrestoBlue®. THP-1 cells were differentiated to THP-1 derived macrophages using 100 ng/ml of PMA for 72 hours. Then, THP-1 derived macrophages were treated with 80 µg/ml of oxidized LDL (oxLDL) and 20 µg/ml of EEP for 6, 24 and 48 hours. The morphological appearance of foam cells was determined by Oil Red O staining while the amount of cholesteryl ester (CE) present in the foam cells were quantified by using Cholestryl Ester Quantitation Assay Kit. Lastly, the level of inflammatory

cytokine secretions, TNF-α and IL-1β secreted by the treated cells were measured by ELISA. Propolis extract of *Geniotrigona thoracica* sp. was found to have high abundance of triterpenoids and the presence of caffeic acid, *p*-Coumaric acid and quercetin compounds via GC-MS and HPLC analysis respectively. EEP from Colony 2 was selected to be used in this study as it has higher abundance of bioactive compounds. The EC_{90} of EEP was determined to be at 20 µg/ml and this concentration was used for subsequent experiments. Morphological observation showed that lipid droplets were reduced in the cytosol of propolis and oxLDL treated THP-1 derived macrophages. Similarly, CE contents were significantly reduced in the combination of oxLDL and EEP treated THP-1 derived macrophages at all time points. In addition, EEP also was found to significantly reduced TNF-α and IL-1β secretions in the supernatant of the combination of oxLDL and EEP treated THP-1 derived macrophages. As a conclusion, EEP from *Geniotrigona thoracica* sp. inhibit the formation of THP-1 derived macrophage foam cells by suppressing secretion of TNF-α and IL-1β which normally be up-regulated during the pathogenesis of atherosclerosis.

CHAPTER 1

LITERATURE REVIEW

1.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arteries initiated by the retention, oxidation and modification of lipid occur at susceptible sites in major conduit arteries (Insull, 2009). Atherosclerosis is a disease characterized by the deposition of excessive fatty acid substances in the arterial intima. The deposition of the of the fatty acids also known as plaque are made up of lipid, cholesterol, calcium and other substances found in the blood stream that causes the arteries to become harden and narrow as the plaque builds up progressively. Atherosclerosis develop progressively through the continuous changes of the arterial wall lesions accompanied with complex inflammatory response. This alarming condition will restrict the flow of blood to vital organs such as heart, brain as well as other parts of the body, which eventually leads to cardiovascular diseases (CVDs) such as myocardial infarction, ischemic stroke, deep vein thrombosis and pulmonary embolism (Sundbøll et al., 2017). Cardiovascular disease (CVD) is the name for the group of disorders of the heart and blood vessels.

1.1.1 Epidemiology and risk factor of atherosclerosis

According to World Health Organization (WHO), in 2017, CVDs are the number one cause of death globally followed by cancer, respiratory diseases and diabetes. It is estimated that 17.7 million people died from CVDs in 2015 that represent 31% of all global deaths, which account for 7.4 million deaths that were due to coronary heart disease and 6.7 million deaths due to stroke. In addition, according to American Heart Association, the number of people diagnosed with heart failure is

projected to rise by 46 percent by 2030 due to the global increase in diabetes and obesity problems. In developing countries, CVDs cause twice as many deaths as HIV, malaria and TB combined. It is also estimated that about 40 to 50% of all cardiovascular deaths are sudden cardiac deaths (SCDs) and the survival rate form sudden cardiac arrest is less than 1% worldwide and close to 5% in the US (Mehra, 2007; Rahman and Woollard, 2017). In Malaysia, the cardiovascular disease epidemic showed that two third of the population in this country had at least one cardiovascular risk factor and at least one third had two or more risk factors (Nuur Amalina et al., 2012). There are two known risk factors of atherosclerosis, which are life styles and conditions. Life styles includes cigarette smoking, unhealthy diet and alcohol consumption (Patel and Rutherford, 2017). On the other hand, for conditions, there a few conditions that can lead to atherosclerosis such as high cholesterol content in blood, high level of low density lipoprotein (LDL), insulin resistance, hyperglycemia (high blood sugar), high blood pressure and obesity (Christou et al., 2005; Tian et al., 2010). Apart from that, according to current research by Gupta et al., 2013, there are also several emerging risk factors of atherosclerosis such as C-reactive protein (CRP), fibrinogen and homocysteine.

1.1.2 Pathogenesis of atherosclerosis (atherogenesis)

Atherosclerosis is characterized by several events including endothelial dysfunction, vascular inflammation and the accumulation of modified lipid, inflammatory cells and cell debris in plaques within the vascular wall. Endothelial cells, leukocytes and intimal smooth muscle cells (Glass and Witztum, 2001).

1.1.2(a) Endothelial dysfunction or injury

A normal and healthy artery consists of three distinct layers, which are tunica intima, tunica media and tunica adventitia (Gimbrone and García-Cardeña, 2013) (Figure 1.0a). Tunica intima or also known as endothelial cell (EC) layer is a monolayer of endothelial cells that lines the lumen of all blood vessels. Tunica media is the central layer of vascular smooth muscle cells (VSMC), elastin fibers and extracellular connective tissue (proteoglycans and collagen) matrix that control vascular tone. Meanwhile, tunica adventitia is the surrounding layer of connective tissue, containing mast cells, dendritic cells, monocytes/macrophages, T cells and micro-vessels or vaso vasorum that provide nutrition to arteries and veins that is related to perivascular adipose tissue which is a vital site for inflammation and angiogenesis (Douglas and Channon, 2014). Endothelial cells that layers the intima function as interface between circulating blood and the rest of the vessel wall and any changes in EC phenotype will affect the function of the arteries via the regulation of Nitric Oxide (NO) which is one of the most significant signaling molecules produced by the endothelium (Bauer and Sotníková, 2010). Nitric oxide play an important role in inducing vasodilation in response to platelet aggregation, reducing adhesion molecules expression to prevent macrophage infiltration and slows the proliferation of VSMC which are protective against atherosclerosis (Vanhoutte, 2009).

The EC dysfunction occurs by several mechanisms such as free radical oxidation (Vanhoutte, 2009). The dysfunctional EC will reduce the production of NO and increased generation of reactive oxygen species (ROS) or superoxide radicals. The synergistic interaction between NO and ROS will form peroxynitrite ($ONOO^-$) that cause damage to proteins and lipids in the arteries leading to alteration of vascular integrity, reduction of vasodilator capacity and appearance of endothelial dysfunction

(Douglas and Channon, 2014). Excess production of ROS is highly pathological and relates directly to atherogenesis.

1.1.2(b) Fatty streaks formation

An increase in the level of low density lipoprotein (LDL) cholesterol is one of the most prominent risk factor in atherosclerosis. LDL is responsive to oxidation by enzymes namely myeloperoxidases, lipoxygenases, NADPH oxidases and nitric oxide synthases resulting in the formation of oxidised LDL (oxLDL) (Tsimikas and Miller, 2011). OxLDL display a cytotoxic effect where it activate and cause injury to the endothelial resulting in an inflammatory response that leads to recruitment, activation and migration of monocytes through inter-endothelial gaps to the sub-endothelial region or arterial intima (Ross, 1999). OxLDL cause endothelial activation via induction of cell surface adhesion molecules, monocyte chemo attractant protein-1 (MCP-1) that mediates the attraction and attachment of monocytes (Chen and Khismatullin, 2015).

Apart from that, oxLDL also stimulates EC to increase the expression of vascular adhesion molecules-1 (VCAM-1) and intracellular adhesion molecules-1 (ICAM-1) that binds to the cell surface receptor of monocytes for adhesion and recruitment (Kim et al., 2001) (Figure 1.0b). According to Lawrence, 2009 , oxLDL was found to activate the generation of nuclear factor kappa B (NF-kB) that is important in modulating expression of pro-inflammatory cytokines (TNF-α, IL-1β), adhesion molecules and chemokines that take part in pathogenesis of atherosclerosis. In tunica intima, blood circulating monocytes undergo differentiation into macrophages under the influenced of macrophage-colony-stimulating-factor (MCSF) as it promotes synthesis of scavenger receptor protein on the surface of the monocytes (Hume and MacDonald, 2012). The differentiated macrophages then will ingest

oxLDL via endocytosis by CD36 scavenger receptor (Silverstein and Febbraio, 2009) resulting in transformation of macrophages into foam cells. The accumulation of foam cells will lead to fatty streaks formation which is the first stage in atherosclerotic plaque development (McLaren et al., 2011). Foam cells contain a massive amount of oxLDL that is composed of esterified cholesterol and triglycerides together with the fatty acids tails of the phospholipid.

1.1.2(c) Atherosclerotic plaque and thrombosis

Atherosclerotic inflammation causes macrophages to be trapped in the lesion as macrophage loss its polarity induced by oxLDL via the activation of Vav/Rac pathway (Chi and May, 2009). Several cell types including SMC, EC, macrophages, monocytes and platelets play a pivotal role in atherosclerotic plaque stability. The plaque is located beneath a fibrous cap of extracellular matrix (ECM) and VSMC and its stability depends on the unimpaired connection between endothelium and fibrous cap that isolate the atherosclerotic lesion from the circulating blood as shown in Figure 1.0c (Douglas and Channon, 2014). In contrast, the plaque will become unstable due to a vast changes in cellular composition and structure causing a mechanical weakness to the fibrous cap leading to platelet aggregation and thrombus formation (Badimon and Vilahur, 2014) (Figure 1.0d). The characteristic of an unstable atherosclerotic plaque include a large lipid core, thin fibrous cap that contain high ratio of macrophages to VSMC and reduced collagen content. These characteristic of unstable plaque are due to the increased in secretion of pro-inflammatory cytokines and the generation of matrix metalloproteinases (MMPs) that degrade collagen produced by VSMC in the fibrous cap causing it to become thinner which then lead to thrombosis (Rundhaug, 2005)

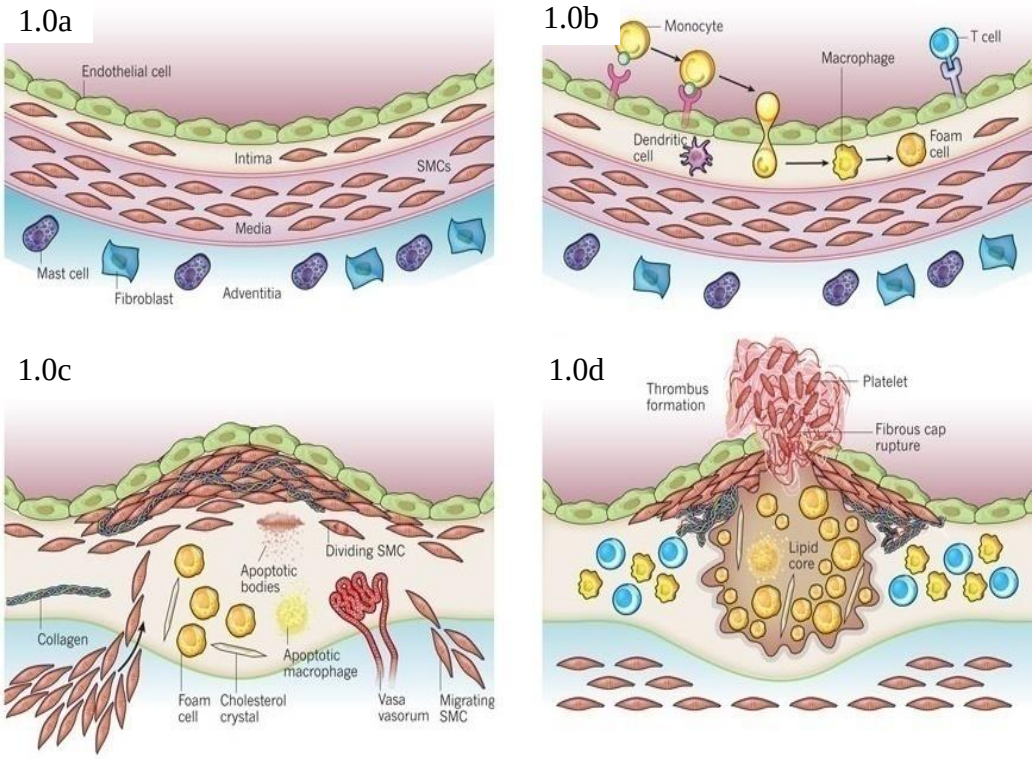

Figure 1.0: Stages in the development of atherosclerotic lesions. **a)** The normal artery contains three layers namely tunica adventitia, tunica media and tunica intima. **b)** Atherogenesis initiated by the adhesion of blood leukocytes to activated endothelial monolayer, facilitate the migration of bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid which convert the mature macrophages into foam cells. **c)** Lesion progression involves the migration of SMC from media to intima, the proliferation of SMC and the increased in synthesis of extracellular matrix macromolecules. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque denoted as necrotic core. **d)** Thrombosis caused physical disruption of the atherosclerotic plaque as the burst of the plaque's fibrous cap will cause blood coagulation components to come into contact with tissue factors in the plaque's interior causing the thrombus to extend into vessel lumen that can block blood circulation (Libby et al., 2011).

1.1.2(d) Current treatment of atherosclerosis

Atherosclerosis is a multi-factorial inflammatory disease and there are various approaches that have been studied and attempted for its prevention and treatment. The strategies used to combat atherosclerosis include lipid lowering drug, anti-inflammatory drug and natural or herbal remedies. The usage of lipid lowering drug namely Statin exhibit anti-inflammatory mechanism that help in reducing cholesterol synthesis by inhibiting HMG-CoA reductase enzyme involve in cholesterol production (Charo and Taub, 2011). Although statin has been used widely throughout the globe for the treatment of atherosclerosis, there is a side effect reported as it can induced rhabdomyolysis that damages skeletal muscle which is life threatening (Sakamoto and Kimura, 2013).

On the other hand, herbal medicines have been traditionally used since ancient time for treatment of various chronic diseases by indigenous herbal practitioners. Herbal extracts or a combination of several herbal supplements are prescribed to patients to improve therapeutic efficacy (Zeng et al., 2012). Herbal plants contain a variety of chemical constituents that work individually or synergistically which contribute in increasing or inhibiting the desired effect for a particular disease (Sasidharan et al., 2011). There are various studies conducted in relation of using natural products for the prevention and treatment of atherosclerosis and among them is the usage of propolis. Propolis has attracted interest of researchers all over the world due to the potency of its biological properties such as anti-inflammatory and anti-atherogenic.

1.2 Propolis: The properties and chemical composition

Propolis is the term used to describe the sticky resinous material collected by variety species of bees. It is also generally known as bee glue comprises of exudates or young shoots and bud of certain plants collected at the surrounding of bee hive that are mix together with wax and bee enzyme (Bankova et al., 2014). The plants secrete the sticky substances in order to coat the young shoots and buds to protect them from abysmal weather as well as from the attack by bacteria, fungi, molds and viruses (Sahinler and Kaftanoglu, 2005). Depending on the origin of the propolis, the color varies from green, red to dark brown with a characteristic smell and shows adhesive properties (Burdock, 1998). Propolis in its natural from is shown in Figure 1.1.

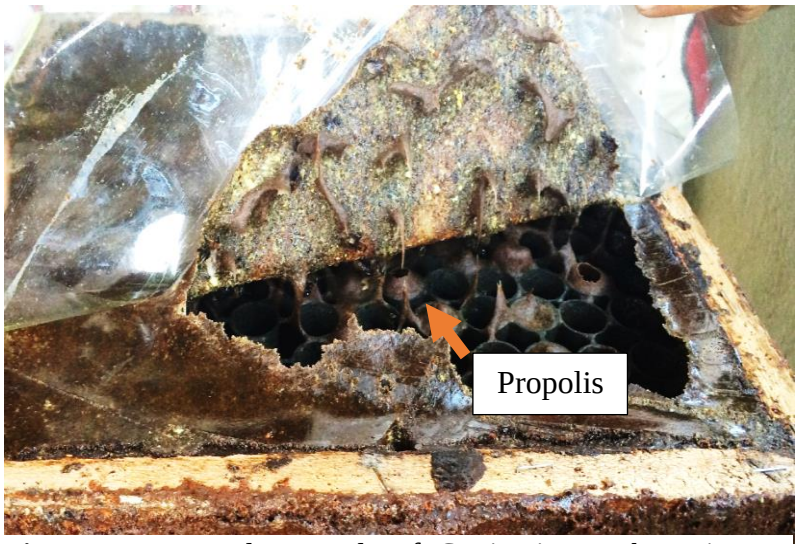

Figure 1.1: Propolis sample of *Geniotrigona thoracica* sp. before being harvested

Propolis serve a variety of functions that benefit both the bee's colony and its hive. The bees collect the resinous substances, transport them using their hind legs, and bring it back to their hive. The propolis will then help to cover cracks and crevices to smooth out the internal walls in the hive as well as reducing the size of the hive entrance to prevent large insects such as butterflies, beetles and moths to enter (Denisow and Denisow-Pietrzyk, 2016). Apart from that, propolis also was used to

seal holes in their honeycombs in order to protect the bee's colony and larvae from pathogenic microorganism such as bacteria and fungi thus helps in maintaining their longevity (Sforcin, 2007). Propolis also function as thermal isolation as it help in keeping the temperature and moisture in the hive at an optimal level throughout the year (Huang et al., 2014). Last but not least, bees also use propolis to cover carcasses of hive invaders such as ants that have died inside the hive to prevent their decomposition which can harm the bees with presence of decomposers in the hive (Burdock, 1998). The word propolis was originated from the combination of two Greek words which are 'Pro' that means in defense, and 'Polis' which means city that subsequently reflect the importance and function of the propolis itself.

Bees modify propolis by using glucodiases enzyme secreted from hypopharyngeal glands during collection and processing. The specific enzymatic modification towards propolis resulted in the hydrolyzation of phenolic compounds like flavonoid heterosides to free flavonoid aglycones and sugars that further enhance the pharmacological action of propolis (Najafi et al., 2007).

Besides that, propolis has long been used extensively as medicine since ancient times in many parts of the world. Propolis also was recognized by the Greek and Roman physicians including Aristoteles, Dioscorides, Pliny and Galen for its beneficial medicinal properties towards human being. According to Aristoteles, propolis was referred as a substance which the bees smeared at the hive entrance and used as a cure for bruises and sores (Bogdanov and Stefan, 2014). In the Middle East, propolis was used among Arab physicians as an antiseptic and cicatrizant in wound treatment as well as mouth disinfectant due to its potent anti-bacterial properties. In addition, the London pharmacopoeias of the seventeenth century had listed propolis as

an official drug to be used for medicinal purposes and it became very popular in Europe due to its anti-bacterial activity (Castaldo and Capasso, 2002).

Apart from that, propolis possesses a wide range of biological and pharmacological properties that has been significantly reported all over the world such as immunomodulatory (Chan et al., 2013) , anti-inflammatory (Naito et al., 2007), antioxidant (Kurek-Górecka et al., 2014), antibacterial (Oliveira et al., 2010), antiviral (Schnitzler et al., 2010) and antifungal (Ota et al., 2001). Therefore, due to the vast medical attributes that propolis showcase, it is currently used as a popular remedy in variety of form such as capsules, extract, mouthwash, in throat lozenges, creams and powder to be used by a wider range of health conscious consumer all over the world (Castaldo and Capasso, 2002). The wide application of propolis in modern medicine has drawn attention and pique interest of researchers to investigate its chemical constituents that contribute to its biological properties.

There are numerous research conducted about propolis and its respective compounds up to this day. There were more than 250 individual compounds that have been established as the constituents of propolis. Natural or raw propolis composed of approximately 50% resin and plant balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and other organic substances. The organic substances present in raw propolis include vitamins, minerals, sugars, alcohols and amino acids (Salatino et al., 2005; Huang et al., 2014). In addition, the usage of separation and purification techniques such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography Mass Spectrometry (GC-MS) allow more compounds to be identified in propolis extract including flavonoids, terpenes, phenolics, sugars and hydrocarbons (Huang et al., 2014). Furthermore, among these organic compounds, phenolics, flavonoids and esters are found to be the most abundant compound present in all their

forms in propolis extract. Chemically, flavonoids compound present in propolis are flavones, flavonoles, flavonones, dihydroflavonoles, isoflavones, neoflavonoids and chalcones. Meanwhile, phenolic compounds include phenolic aldehydes and phenolic derivatives of cinnamic and benzoic acid such as caffeic acid esters, terpenes, steroids, sesquiterpenes, naphthalene and stilbene terpenes (Marcucci et al., 2001; Najafi et al., 2007).

Phenolic compounds are substances that are found mainly in fruits as they contribute to its color and taste. Chemically, phenols are made up of an aromatic ring bound with one or more hydrogenated substituents including their functional derivatives (Izawa et al., 2011). Phenolic compounds comprises of a variety of groups including phenolic acids, phenolic aldehydes, benzoic and cinnamic acid and their derivatives, coumarins and phenols and their esters. The most common derivatives of benzoic and cinnamic acid are caffeic acid and *p*-Coumaric acid respectively (Kurek-Górecka et al., 2014). Not just that, phenolic compounds in their variety of forms are reported to be responsible for many biological attributes such as antioxidant (Chirinos et al., 2013), antibacterial (Inui et al., 2014), anti-inflammatory (Park, 2011) and anti-atherogenic properties (Daleprane et al., 2012)

Flavonoids are a group of natural compounds synthesized via phenylpropanoid pathway that are found ubiquitously in plants. The chemical structure of flavonoids consists of a 15-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1.2 linked via a heterocyclic pyrane ring (C). Flavonoids can be divided into different classes that differ in the level of oxidation and pattern of substitution of the A and B rings. The different classes of flavonoids are flavones such as apigenin and luteolin; flavonols such as quercetin, kaempferol, myricetin and fisetin; flavonones such as hesperetin and naringenin (Kumar and Pandey, 2013).

As one of the major constituents of propolis, flavonoids play a major role in the biological properties of propolis. It was reported that flavonoids contribute to the activities such as antibacterial (Cushnie and Lamb, 2011), antiviral (Özçelik et al., 2011) and anti-inflammatory (Pan et al., 2010). Flavonoids also was found to have protective effect against degenerative diseases such as cardiovascular diseases (CVDs) and cancers (Cook and Samman, 1996). Flavonoids was reported to display antiatherogenic effects by breaking the chain reaction of lipid oxidation initiated by free radicals which help to protect lipoprotein from being oxidized thus reduce the risk factor of atherosclerosis (Kurek-Górecka et al., 2014).

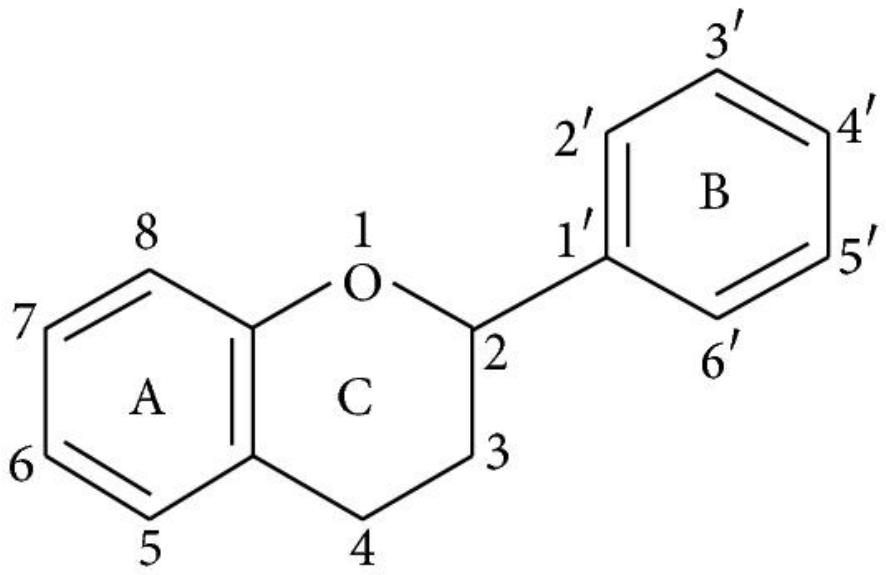

Figure 1.2: Chemical structure of flavonoids. The chemical structure of flavonoids consists of a 15-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1.2 linked via a heterocyclic pyrane ring (C). (Kumar and Pandey, 2013)

1.2.1 Biological activities of propolis and its contributing constituents

There were numerous study on biological properties of propolis. The biological properties such as immunomodulatory, anti-inflammatory, antioxidant, antibacterial and antiviral are among those that had been reporetd. The wide application of propolis is due to its biological attributes that might be the result of synergistic action of its complex constituents such as flavonoids, phenolic, terpenoids, steroids, amino acids and many other constituents (Huang et al., 2014) as shown in Table 1.0.

Table 1.0: Biological activities of propolis and contributing constituents (Farooqui and A Farooqui, 2010).

Activity	**Constituent**
Antimicrobial	Terpenes: diterpenes, triterpenes
Antibacterial	Chrysin, apigenin, pinocembrin and galangin
Antiviral (anti-influenza virus and anti-herpes simplex virus type 2)	Polyphenols, flavonoids, phenyl-carboxylic acids and esters of substituted cinnamic acids (caffeic acid, *p*-coumaric acid, benzoic acid, galangin, pinocembrin).
Anti-oxidant	Caffeic Acid Phenolic Ester (CAPE), caffeic acid, quercetin, kaempferol, luteolin, chrysin, propolins, polyisoprenylated benzophenone, artepillin C, caffeoylquinic acid derivatives.
Anti-inflammatory	CAPE, quercetin, chrysin
Immunomodulatory	CAPE
Hepatoprotective	CAPE, chrysin, diterpenes
Cardioprotective	CAPE, acacetin, chrysin, quercetin
Anticancer	CAPE, artepillin C, chrysin, quercetin, propolin C
Antitumor	Artepillin C, caffeic acid, CAPE, quercetin, cinnamic acid derivatives, baccharin, drupanin, propolins
Anti-ulcer	Caffeic, ferulic, p-coumaric and cinnamic acids, essential oil

1.2.2 Variation in propolis properties from different geographical region

Propolis possess remarkable biological properties due to its vast chemical compositions and both of the factors directly correlates with the geographic location or site where the propolis are produced. This is due to the fact that in different ecosystem, stingless bees collect resins from different type of plants with respect to the local flora in that particular area (Bankova et al., 2014). Chemical studies performed on propolis to identify their specific chemical constituents from different geographical region also show a high degree of variability (Bankova, 2009). For instance, in temperate zones such as Europe, North America and the non-tropical regions of Asia, the main source of plants used for bees to produce propolis are from the bud exudates of *Populu*s species or poplar trees and their hybrids (Greenaway et al., 1987; Bankovana et al., 2000). However, in tropical regions, poplar trees do not grow in the climate, hence the stingless bees must find other alternatives to produce propolis. For instance, in Venezuela, stingless bees used Clusia major and Clusia minor (Guttiferae) as the primary sources of exudates to generate propolis which is definitely different with the sources used in temperate regions (Tomás-Barberán et al., 1993).

The difference in the source of plants used by stingless bees have shown to affect the chemical composition of the propolis itself. Propolis from Europe and Argentina contain high levels of chrysin, pinocembrin, pinobaksin-acetate and galangin (Gardana et al., 2007). Meanwhile, propolis originated from Taiwan and Japan are highly abundant with propolins and prenylated falvonone compounds (Chen et al., 2004; Kumazawa et al., 2007). The differences in chemical composition caused difficulties in the standardization of propolis extract. Hence, according to Bankova, 2005 the biological properties of each propolis must be determined based on its

detailed investigation of chemical composition and to its related botanical sources. In addition, comparative studies have proven that propolis always exemplify similarities in its biological activity regardless of their differences in chemical composition (Seidel et al., 2008).

Table 1.1: Most widespread propolis type, geographical origins and their plant sources (Sforcin and Bankova, 2011; Toreti et al., 2013).

Propolis type	**Geographical origins**	**Plant source**
Poplar	Europe, North America, Bulgaria, Albania, South Brazil, Argentina, Uruguay, Mongolia non-tropic regions of Asia, New Zealand	*Populus* sp. of section *Aigeiros, P. italic, P. tremula, P. suaveolens, P. fremontii, P. euramericana, P. alba,*
Green (alecrim) Brazilian	Brazil	*Baccharis* sp. predominantly *B. dracunculifolia*
Birch	Russia, Hungary and Poland	*Betula verrucosa*
Red propolis	Cuba, Brazil (type 13 Brazillian, northeast Brazil) and Mexico	*Dalbergia* sp.
Mediterranean	Sicily, Greece, Crete, Malta	*Cupressaceae* (species unidentified)
Clusia	Cuba, Venezuela and other equatorial regions	*Clusia* sp. or *Clusia minor*
Pacific	Pacific region (Okinawa, Taiwan, Indonesia)	*Macaranga tanarius*

1.2.3 Diversity of stingless bees species in Malaysia

Stingless bees belong to a large group of bees in the subfamily of Apidae in the Meliponi tribe of the Hymenoptera order of insects. Stingless bees are social bees that are able to produce propolis collected from buds and exudates of plants and honey by collecting and storing nectar in a honey pot. There are more than 500 species of stingless bees that have been identified globally (Kelly et al., 2014). The stingless bees are prone to be found in tropical countries such as Malaysia, Brazil, Cuba, Venezuela and Cambodia. The stingless bees species that are found in Malaysia varies between 17 to 32 species that are closely related with the area of study (Salim et al., 2012). The species that can be found in Malaysia are *Trigona itama, Trigona thoracica, Thrigona apicalis, Trigona terminata, Trigona respani, Trigona melanocephala, Trigona valdezi, Trigona collina, Trigona atripes, Trigona canifrons, Trigona iridepennis* and *Trigona rufibasalia* (Mohd et al., 2010).

The cultivation or beekeeping of stingless bees are commonly known as meliponiculture, which are done for commercialization purposes. The stingless bees sp. that are most widely used in meliponiculture in Malaysia are *T. thoracica* and *T. itama* (Kelly et al., 2014). In Malaysia, meliponiculture are extensively done throughout the nation and the industry was already a 200 million ringgit a year in profit (Mohd Saufi and Thevan, 2015). Each stingless bees hive produced about 1.5 kg to 2.0 kg of honey per month that are sold between RM150 to RM 200 per kilogram. The bee's hives also is a source of propolis and beebread, which had been used commercially in the health and cosmetics industries (Iryani and Ismail, 2016).

1.2.4 Characteristics and behavior of *Geniotrigona thoracica* sp. of stingless bees

Geniotrigona thoracica sp. of stingless bees are noticeably small, about 2 to 14 mm long with relatively absence of hair and round shape abdomen. It is black in color with four membranous transparent brownish wings and buccal parts that form a tongue-like structure which function in food intake. It also has a highly modified hind leg similar to *T. itama* sp. with atrophied sting but the bees are highly likely to bite if their nest is disturbed (Sakagami and Inoue, 1989). *Geniotrigona thoracica* sp. of stingless bees are active all year round collecting nectar and pollen from different plants (Biesmeijer et al., 2006). Apart from that, every species of stingless bees has specific nest preferences according to their sizes, population and habitat quality (Couvillon et al., 2008). *Geniotrigona thoracica* sp. of stingless bees are prone to make a nest at a tree with a trunk circumference ranging from 82 cm to 129 cm. The nest entrance of stingless bees that are found to be enhance with propolis are also different depending on species as it function in physical defense against invaders and physio-chemical regulation (Lima et al., 2013). *Geniotrigona thoracica* sp. of stingless bees form a mount-shape entrance with the widest entrance compared to all other species with a length 3.97 cm and 4.0 cm in width.

Figure 1.3: *Geniotrigona thoracica* sp. of stingless bee morphology

Figure 1.4: The nest entrance of *Geniotrigona thoracica* sp. of stingless bees in the shape of mount.

1.3 Immunomodulatory action of propolis

Propolis possess anti-atherogenic and anti-inflammatory activities that is related to the progression of atherosclerosis. According to Orsatti et al., 2010, propolis has shown to exert an effect or influence on the immune system particularly during inflammation. Propolis demonstrate anti-atherogenic properties by down-regulating pro-inflammatory cytokines such as MCP-1, IFN-γ, IL-6, TGF-β and VCAM-1 expression in both initial atherosclerotic lesions and advanced atherosclerotic lesions mice (Daleprane et al., 2012). Cytokines play a very important role in enhancing the local inflammatory response, which promotes the progression of atherosclerotic lesions. Hence modulating cytokines expression that involve in inflammation process is the key to prevent atherogenesis. Other than that, MMPs which is known as one of the most important angiogenic biomarkers in atherosclerotic plaque development is

shown to be significantly down-regulated by propolis that can be seen in the decreased in atherosclerotic lesion in both group of treated mice (Daleprane et al., 2012).

Besides that, propolis exert anti-inflammatory activity by suppressing the gene activation of NO synthase and TNF-α mediated NF-κB activation through the presence of caffeic acid phenetyl ester (CAPE) which is one of the active constituents of propolis (Farooqui and Farooqui, 2010; Márquez et al., 2004; Song et al., 2002). CAPE inhibits the enzyme activities of COX-1 and COX-2 and suppresses the transcriptional expression of COX-2, resulting in diminished synthesis of prostaglandins, major mediator of inflmammation. Apart from that, CAPE was found to exert its inhibitory effect on NF-κB by reducing the reactive oxygen intermediates production that is needed for NF-κB activation (Natarajan et al., 1996). Propolis was reported to exert immune response through macrophage activation by inducing the release of hydrogen peroxide and reduce NO production by inhibiting inducible NO synthase (i-NOS) expression and its catalytic activity (Araujo et al., 2012).

1.4 THP-1 cell line application in immune modulation approach

THP-1 cell is a human leukemia monocytic cell line that was isolated from the whole blood of a 1 year old boy that suffer acute monocytic leukemia by Tsuchiya et al., 1980. THP-1 cell has become one of the most important and widely used cell line in the field of immunology particularly in the research regarding the function and regulation of monocytes and macrophages in cardiovascular system. This is due to the fact that, THP-1 cells have a very close resemblance to primary monocytes and macrophages in morphological and functional properties as well as in its differentiation markers (Kramer and Wray, 2002; Ueki et al., 2002). THP-1 cell show a round-single cell morphology that express distinct monocytic markers (Chanput et

al., 2015). Apart from that, THP-1 cell line is a type of immortalized cell line as it can be cultured in vitro for up to passage 25 without any changes in cell morphology and metabolic activity which benefit researchers as the cell stability is well-preserved (Chanput et al., 2014). Besides that, the usage of THP-1 cell line reduce the degree of variability in the cell phenotype due to its homogenous genetic background which provide advantage over the primary monocytes or macrophages in immune modulation approach (Aldo et al., 2013)

The human monocytic cell line THP-1 can also be differentiated into THP-1 derived macrophages, which display the same metabolic and morphological appearances. There are various differentiation agents used to differentiate THP-1 cells such as phorbol-12-myristate 13 acetate (PMA) and 1,25-dihydroxyvitamin D3 (VD3) (Daigneault et al., 2010a). Most of the THP-1 cells shown to adhere to culture plate as it start to change its morphology into flat and amoeboid shape with well-developed golgi apparatus, rough endoplasmic reticulum and ribosomes in the cell's cytoplasm after the exposure to PMA as it undergoes differentiation process that display mature macrophages properties (Chanput et al., 2014). PMA treatment towards THP-1 cells activates protein kinase C (PKC) inducing a greater degree of differentiation (Daigneault et al., 2010a). PMA treated THP-1 cells was reported to generate a more mature macrophages phenotype with higher level of adherence, lower proliferation rate, higher phagocytic activity and a higher expression of CD11b, a cell surface marker of macrophages (Qin and Zhenyu, 2012; Schwende et al., 1996). In addition, according to Qin and Zhenyu, 2012, THP-1 cell and the differentiated THP-1 derived macrophages mimic the *in situ* alteration of monocyte and macrophages in atherosclerotic lesion which make it suitable to be used in the study of physiology and pathophysiology of cardiovascular diseases. Finally yet importantly, THP-1 cells are

the most widely used human monocyte-macrophages cell line in research related to atherosclerosis.

1.5 Rationale of study

Atherosclerosis is a worldwide epidemic and researchers must take action to find ways to reduce the risk factors contributing to it and come up with an appropriate treatment that poses no harm or adverse effect to human being. The pathogenesis of atherosclerosis involve specific immune response elicited by various effector cells that secrete numerous cytokines and enzymes and the proposed treatment for atherosclerosis must focus on a particular cell's secretion such as pro-inflammatory cytokines in order to combat atherosclerosis.

The usage of herbal or natural product is one of the suggested solution and propolis was utilized in this research to investigate its action on specific cells and its secretion. Propolis from different species in various geographic regions were reported to possess many biological attributes including anti-atherogenic contributed by its complex chemical constituents. However, to the best of our knowledge, there is no study conducted on propolis produced by Indo-malayan stingless bees particularly from *Geniotrigona thoracica* sp. of stingless bees. Indo-malayan stingless bees or lebah kelulut is one the most common type of bees found in Malaysia and the utilization of its bees product such as propolis in terms of scientific research for its therapeutic value particularly in atherosclerosis will give benefit to a wide range of people. Therefore, this study was conducted specifically to investigate the immunoregulatory role of propolis from *Geniotrigona thoracica* sp. of Indo-malayan stingless bees on the formation of foam cells in atherosclerosis development.

1.6 Objectives of study

This study was conducted to investigate the effect of ethanolic extract of propolis (EEP) from *Geniotrigona throracica* sp. of stingless bees on the formation of THP-1 derived macrophage foam cells.

The objectives of this study are listed below:

1. To determine the bioactive compound of EEP from *Geniotrigona throracica* sp. of stingless bees.
2. To determine the effect of EEP on the secretion of inflammatory cytokines by oxLDL treated macrophages.
3. To determine the effect of EEP on the formation of lipid laden foam cells derived from oxidized LDL-treated macrophages.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and suppliers

The materials, antibodies, reagents, chemicals and commercial kits used in the study were listed in Table 2.1.

Table 2.1 List of materials used in the study

Materials	**Brand, Origin**
Cells: THP-1 cells	 ATCC, USA
Cell Culture Media: Roswell Park Memorial Institute (RPMI) 1640 Medium	Gibco BRL, UK
Antibody: APC-Cy 7 Mouse Anti-Human CD11b PE Rat Anti-Human CD115	 BD Pharmingen, USA BD Pharmingen, USA
Reagents: Fetal Bovine Serum (FBS) Penicillin/Streptomycin Oxidized Low Density Lipoprotein (oxLDL), Human Phorbol 12-myristate 13-acetate (PMA) Presto Blue Viability Reagent Staining buffer	 Gibco BRL, UK Gibco BRL, UK Alfa Aesar, Thermo Fisher Scientific, UK Sigma Aldrich, USA Thermo Fisher Scientific, USA BD Pharmingen, USA BD Pharmingen, USA
Chemicals: Oil Red O Haematoxylin 2-propanol Formaldehyde Ethanol Dimethyl sulfoxide (DMSO) Methanol Acetonitrile Formic Acid Baicaline Hesperetin *p*-Coumaric Acid Caffeic acid	 Amresco, USA R&M Chemicals Ltd., USA QRec Asia Amresco, USA HmbG Chemicals, Germany Sigma Aldrich, USA Fisher Scientific, USA Fisher Scientific, USA Fisher Scientific, USA Toronto Chemicals, Canada Toronto Chemicals, Canada Toronto Chemicals, Canada Toronto Chemicals, Canada

Table 2.1: Continued

Kaempferol Quercetin Naringenin	Toronto Chemicals, Canada Toronto Chemicals, Canada Toronto Chemicals, Canada
Commercial Kit: Cholesterol/Choletsryl Ester Quantitation Kit Human IL-1 beta ELISA Ready-Set-Go (2^{nd} Generation kit Human TNF alpha ELISA Ready Set-Go Kit	 Abcam, UK Affymetrix, eBioscience, USA Affymetrix, eBioscience, USA