UNIVERSITI TEKNOLOGI MARA

GC-MS ANALYSIS OF PHYTOCHEMICALS AND EVALUATION OF ANTIOXIDANT AND ANTI-ADIPOGENIC ACTIVITIES OF GENIOTRIGONA THORACICA PROPOLIS FROM DIFFERENT LOCATIONS IN MALAYSIA

LINI BINTI IDRIS

MSc

March 2026

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of the requirements for the degree of
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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 23 June 2025 to conduct the final examination of Lini binti Idris on her Master of Science thesis entitled "GC-MS analysis of phytochemicals and evaluation of antioxidant and anti-adipogenic activities of *Geniotrigona thoracica* propolis from different locations in Malaysia" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiner recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

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ABSTRACT

Geniotrigona thoracica is a recognized stingless bee species cultivated in the Malaysian meliponiculture industry. It is known for producing propolis rich in bioactive compounds and exhibiting a range of biological activities. However, the anti-adipogenic potential of Malaysian propolis remains insufficiently studied. This study explores the phytochemical composition, antioxidant properties, and anti-adipogenic activity of ethanolic extract of propolis (EEP) from *G thoracica* hives at three locations in Selangor including Serdang (SER), Shah Alam (SA), and Hulu Bernam (HB). Propolis was extracted using maceration with 70% ethanol. Phytochemical screening using Gas Chromatography-Mass Spectrometry (GC-MS) revealed variations in compound profiles, with 15 distinct compounds identified in the SER extract, 15 in SA, and 12 in HB. SER contained 15 distinct compounds, including phenolics, terpenoids, sugar alcohols, and aromatics. SA showed 15 compounds from similar major classes as SER, while HB presented 12 compounds, including phenolics, terpenoids, steroids, fatty acids, and aromatics. The EEP samples were assessed for total phenolic content (TPC) and total flavonoid content (TFC). Antioxidant activity was evaluated using the Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the Ferric Reducing Antioxidant Power (FRAP) assay. Additionally, the anti-adipogenic effects of EEP were investigated using the 3T3-L1 preadipocyte cell line model, following cytotoxicity screening at various concentrations. Notably, the EEP from SER demonstrated superior antioxidant properties, with the highest TPC (302.21 ± 0.11 mg/mL GAE), TFC (99.08 ± 0.03 mg/mL QE), and FRAP value (727.53 ± 0.09 uM Fe^{2+}), along with the lowest DPPH ICso value (25.27 ug/mL). EEP from all three locations was non-cytotoxic. However, the SER extract demonstrated the most potent anti-adipogenic effects, significantly reducing lipid accumulation and reactive oxygen species (ROS) production. Quantitative real-time PCR (qRT-PCR) further revealed that the SER extract more effectively downregulated key adipogenic transcription factors, including sterol regulatory element-binding protein-1 (SREBP-1), peroxisome proliferator-activated receptor-gamma (PPAR-y), and CCAAT/enhancer-binding protein-alpha (C/EBP-a), highlighting its anti-adipogenic potential. In conclusion, propolis from the SER location exhibits strong antioxidant activity, which may enhance its effectiveness, and is complemented by significant anti-adipogenic properties. This highlights its potential as a natural resource for obesity management. Future research should focus on isolating and characterizing the specific phytochemical components responsible for these effects to further explore their potential applications in health interventions.

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LIST OF SYMBOLS

Symbols

Akt	Protein Kinase B
cDNA	Complementary DNA
-COOH	Carboxyl Group
CO_2	Carbon Dioxide
°C	Degrees Celsius
Fe^{2+}/Fe^{3+}	Iron (II)/Iron (III) ions
g, xg	Gram, Relative Centrifugal Force (g-force)
H_2O_2	Hydrogen Peroxide
hPa	Hectopascal
mRNA	Messenger RNA
nm	Nanometer (wavelength)
•OH	Hydroxyl Radical
-OH	Hydroxyl Group
OH	Hydroxide ion
O_2	Superoxide anion
$P<0.05$	Probability value less than 0.05 (statistical significance)
ug/mL, mg/mL	Microgram per milliliter, Milligram per milliliter
uL, mL, L	Microliter, Milliliter, Liter
uM, mM, M	Micromolar, Millimolar, Molar

LIST OF ABBREVIATIONS

Abbreviations

ATCC	American Type Culture Collection
BAT	Brown Adipose Tissue
BMSCs	Bone Marrow Mesenchymal Stem Cells
CAPE	Caffeic Acid Phenethyl Ester
C/EBP (a, P)	CCAAT/Enhancer-Binding Protein (alpha, beta)
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-l-picrylhydrazyl
EEP	Ethanolic Extract of Propolis
FABP4	Fatty Acid-Binding Protein 4
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Power
G. thoracica	*Geniotrigona thoracica*
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
HB	Hulu Bernam (Sample Location)
HFD	High-Fat Diet
HPLC	High-Performance Liquid Chromatography
IBMX	3 -Isobutyl-1 -methylxanthine

IC_{50}	Half Maximal Inhibitory Concentration
IL-6	Interleukin-6
MAPK	Mitogen-Activated Protein Kinase
MARDI	Malaysian Agricultural Research and Development Institute
MSCs	Mesenchymal Stem Cells
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-Acetylcysteine
NBT	Nitroblue Tetrazolium
NF-KB	Nuclear Factor Kappa B
NIST	National Institute of Standards and Technology
OD	Optical Density
ORO	Oil Red O
P/S	Penicillin-Streptomycin
PBS	Phosphate-Buffered Saline
PI3K	Phosphoinositide 3-Kinase
PPAR-y	Peroxisome Proliferator-Activated Receptor Gamma
qRT-PCR / RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
QE	Quercetin Equivalent
ROS	Reactive Oxygen Species
SA	Shah Alam (Sample Location)
SD	Standard Deviation
SER	Serdang (Sample Location)

SREBP-lc	Sterol Regulatory Element-Binding Protein lc
SVCs	Stromal-Vascular Cells
TFC	Total Flavonoid Content
TMS	Trimethylsilyl
TNF-a	Tumor Necrosis Factor-alpha
TPC	Total Phenolic Content
TPTZ	2,4,6-Tripyridyl-s-triazine
UCP1	Uncoupling Protein 1
UV	Ultraviolet
WAT	White Adipose Tissue

CHAPTER 1
INTRODUCTION

1.1 Research Background

Stingless bees or locally known as "kelulut" produce honey, bee bread, and propolis (Ngalimat et al., 2020). Propolis is a sticky, resinous substance collected from plant leaves, buds, and exudates (Bobis, 2022). In general, it is composed of 50% resinous compounds, 30% wax, 5% pollens, and 5% minor components (Syed Salleh et al., 2021). It acts as natural defense to protect the colony from predators and to repair the beehives (Chuttong et al., 2023). In comparison to honeybees, stingless bees produce less amount of honey, but they are able to produce substantial quantity of propolis (Ibrahim et al., 2016). Furthermore, stingless bee propolis is perceived to have superior therapeutic properties compared to honeybees (Mohd-Yazid et al., 2018). In Malaysia, *Geniotrigona thoracica* is one of the stingless bee species commonly used in the meliponiculture industry (Kelly et al., 2014).

Previous studies have demonstrated that Malaysian *G thoracica* propolis exhibits high antioxidant activity compared to propolis derived from other stingless bee species (Adli et al., 2022). Polyphenolic compounds are regarded as the major bioactive constituents contributing to the antioxidant, antimicrobial, anti-inflammatory, antifungal, and anticancer properties of propolis (Al-Hatamleh et al., 2020). However, the chemical composition of propolis is highly variable and strongly influenced by floral origin, geographical location, and foraging behaviour of the bees (Bobi§, 2022). Such variability contributes to differences in biological activities among propolis extracts.

Antioxidant, oxidative stress, reactive oxygen species (ROS) and free radicals are commonly used terms in diseases mechanism (Afzal et al., 2023). A free radical is an unstable atomic or molecular orbital that contains one or more unpaired electrons (Chaudhary et al., 2023). The presence of excessive amounts of unstable free radicals, causes the production of ROS, an oxidizing agents produced as a product from cellular metabolism (Martemucci et al., 2022). ROS such as hydrogen peroxide (H2O2), hydroxyl ion (HO-), and superoxide anion (O2-), can be generated through the process

of cellular metabolism, or through the exposure of external factors and exogenous agents like ultraviolet (UV), toxins, and drugs (Jena et al., 2023).

ROS in normal amounts is essential for various physiological processes in defense systems, hormone biosynthesis, gene expression and cellular signalling (Chatterjee, 2016). However, the overproduction of ROS results in a condition known as oxidative stress where it has been shown to induce various diseases such as obesity, heart failure, stroke, diabetes, dyslipidaemia, hypertension, atherosclerosis, and other chronic diseases (Corbi et al., 2014). Oxidative stress is a general term for cellular damage caused by an imbalance between the production of ROS and antioxidant molecules (Chatterjee, 2016). Antioxidants are necessary to maintain the level of ROS since they can transform free radicals into stable molecules and prevent oxidation damage by inhibiting ROS formation within the cells (Kwak et al., 2021).

Obesity is a metabolic disorder characterized by increased adipose tissue mass in the whole body, which is associated with excess fat accumulation in adipocytes (Zhai et al., 2020). Obesity metabolic disorders mostly depend on the abnormally increased of adipose tissue during the process differentiation of preadipocytes into adipocytes (Zorena et al., 2020). The differentiation leads to an increase in the number of adipocytes (hypertrophy) and enlargement of the size of adipocyte (hyperplasia) (Horwitz & Birk, 2023). Adipocyte differentiation can generate high quantity of ROS and lead to oxidative stress (Waheed et al., 2022). Excess oxidative stress in obese patients coincide with fat accumulation in adipose tissue (Masschelin et al., 2020).

Obese individuals exhibit higher levels of oxidative stress in adipose tissue, including elevated ROS levels and decreased antioxidant activity (Swiatkiewicz et al., 2023). Thus, the development of antioxidants could be a valuable approach for treating and preventing the excess adipogenesis formation and its related diseases (Almoraie & Shatwan, 2024). A healthy functional diet that includes radical quenching substances is required to exert a defensive mechanism in order to eliminate excess ROS (Nimse & Pal, 2015). Polyphenol compounds possess high antioxidants and anti-adipogenic activities, and they can protect cells against oxidative damage by removing ROS (Lee etal., 2013).

1.2 Problem Statement

Although propolis has been widely reported to contain high levels of bioactive phytochemicals that contribute to its antioxidant and anti-adipogenic properties, its chemical composition and biological activity are highly variable due to differences in geographical region, botanical origin, and bee species. Most existing studies focus on honeybee propolis, while scientific evidence on stingless bee propolis, particularly that derived from *G thoracica,* remains limited.

Furthermore, comparative studies evaluating both the phytochemical composition and biological activities of stingless bee propolis from different locations are scarce. This lack of systematic investigation limits the scientific substantiation of therapeutic claims associated with stingless bee propolis and hinders its potential application in functional food and nutraceutical development. Therefore, this study aims to screen and compare the phytochemical constituents, antioxidant activity, and anti-adipogenic potential of ethanolic extracts of *G thoracica* propolis collected from three different locations in Selangor, Malaysia.

1.3 Research Objectives

The objectives of this study are:

a) To screen the chemical profiles of EEP from *G thoracica* collected from Serdang, Shah Alam, and Hulu Bernam, Selangor, using Gas Chromatography - Mass Spectrometry (GC-MS).

b) To evaluate the antioxidant activities, including total phenolic content (TPC), total flavonoid content (TFC), DPPH, and FRAP assays of EEP from *G thoracica* collected from Serdang, Shah Alam, and Hulu Bernam, Selangor.

c) To assess the anti-adipogenic activities of EEP from *G thoracica* collected from Serdang, Shah Alam, and Hulu Bernam, Selangor using the 3T3-L1 cells adipocyte model.

1.4 Significance of Study

The research outcome would generate new knowledge in substantiating the therapeutic claim of local stingless bee propolis and hopefully will lead to the potential application as an ingredient for development of functional foods and nutricosmetics for obesity prevention. Furthermore, this study could also help in promoting the commercial value of local stingless bee products and will contribute to the growing body of knowledge on the potential benefits of stingless bee propolis.

1.5 Scope and Limitation

This study focused on the analysis and comparison of propolis produced by *G. thoracica* stingless bees collected from three geographical locations in Selangor, Malaysia, namely Serdang, Shah Alam, and Hulu Bernam. Propolis samples were extracted using 70% ethanol through maceration to obtain ethanolic extracts of propolis (EEP). Phytochemical screening was conducted using Gas Chromatography-Mass Spectrometry (GC-MS) to obtain a qualitative profile of volatile and semi-volatile constituents present in the extracts following derivatization. GC-MS was selected for this study due to its suitability for exploratory screening and tentative identification of diverse compound classes through spectral library matching. However, this technique does not comprehensively detect non-volatile or high-molecular-weight phenolic compounds, therefore, the phytochemical data obtained represent a qualitative profile rather than a complete chemical composition.

Antioxidant activities were evaluated using total phenolic content (TPC), total fiavonoid content (TFC), DPPH radical scavenging, and FRAP assays. Anti-adipogenic activity was assessed through *in vitro* experiments using the 3T3-L1 preadipocyte cell line model.

CHAPTER 2
LITERATURE REVIEW

2.1 Stingless Bees

The stingless bee belongs to the order Hymenoptera within the family Apidae, specifically grouped under the tribe Meliponini (Engel et al., 2023). It can be further classified into two main genera, Melipona and Trigona (Engel et al., 2023). It is known as the smallest bee species in the same family, with sizes typically ranging between 3 to 8 mm, depending on the species (Jaafar, 2012). About 500 species of Meliponini have been described worldwide, and approximately 50 stingless bee species have been identified in Malaysia (Toledo-hernandez et al., 2022). Meanwhile, nearly 40 different species are known to be native to Malaysia (Ivorra et al., 2020). Stingless bee is an eusocial insects that live in colonies and widely distributed across dry and humid tropical rainforests as well as subtropical regions worldwide (Goh et al., 2023).

Stingless bees are ecologically active and play an essential role in the forest ecosystem (Salatnaya et al., 2023). They are major visitors of many flowering plants in the tropics due to its character as polylecty, where they are capable of pollinating multiple plant species (Khalifa et al., 2021). This characteristic makes them highly valuable as pollinators for both wild flora and agricultural crops. A distinctive feature of stingless bees is their reduced or non-functional stinger, which is significantly different from other bees, making them unable to sting as a defense mechanism (Mustafa et al., 2018). Instead, stingless bees rely on biting and the production of defensive substances to ward off threats (Vit et al., 2004).

The term "stingless" is thus used to describe their morphology, distinguishing them from other members of the Apidae family. Due to their non-aggressive nature, stingless bees have gained popularity in commercial beekeeping (meliponiculture) for honey, propolis production, and pollination services (Kelly et al., 2014; Rattanawannee & Duangphakdee, 2019). In Malaysia, meliponiculture has gained traction due to its economic potential and increasing demand for stingless bee products, including honey, pollen, and propolis (Rattanawannee & Duangphakdee, 2019). The growing awareness

of the medicinal and nutritional benefits of stingless bee products has encouraged entrepreneurs to venture into meliponiculture (Majid et al., 2019). Additionally, governmental and non- governmental organizations have been promoting stingless bee farming as a sustainable and environment friendly to engage in meliponiculture activity (Majid etal., 2019).

2.1.1 *Geniotrigona thoracica*

Geniotrigona thoracica is among the most commonly used stingless bee species for commercialization in Malaysia's meliponiculture industry (Jaafar, 2012; Kelly et al., 2014). It is the largest stingless bee species, approximately 8.39 mm long (Samsudin et al., 2018; Ivorra et al., 2020). This species is an endemic species to Malaysia and the most used species for its honey and propolis, due to its superior bioactive properties. Among stingless bees, *G thoracica* is particularly valued for its propolis, which has demonstrated significantly higher antioxidant activity compared to other bee species (Norowi et al., 2010; Badiazaman et al., 2019). Propolis derived from *G. thoracica* has been found to contain a high concentration of bioactive compounds, particularly from the phytochemical classes of polyphenols, and flavonoids (Martinotti et al., 2025).

A study by Adli et al. (2022) revealed that propolis from *G thoracica* exhibited the highest total phenolic compound (TPC) and total flavonoid content (TFC) among five tested stingless bee species. The high phenolic and flavonoid content is structurally associated to its strong bioactivities (Baba & Malik, 2015). The presence of flavonoids such as galangin, chrysin, and quercetin, as well as hydroxycinnamic acids like caffeic acid and its ester caffeic acid phenethyl ester (CAPE), is common to the biological qualities in propolis (Martinotti et al., 2025). These compounds are characterized by phenolic ring structures. For instance, in the case of flavonoids, the basic skeleton structure of flavonoids, Diphenylpropane (C6-C3-C6), has a varying hydroxylation pattern (Figure 2.1.1), which are associated with potent anti-inflammatory, antioxidant, and antimicrobial properties (Chagas et al., 2022).

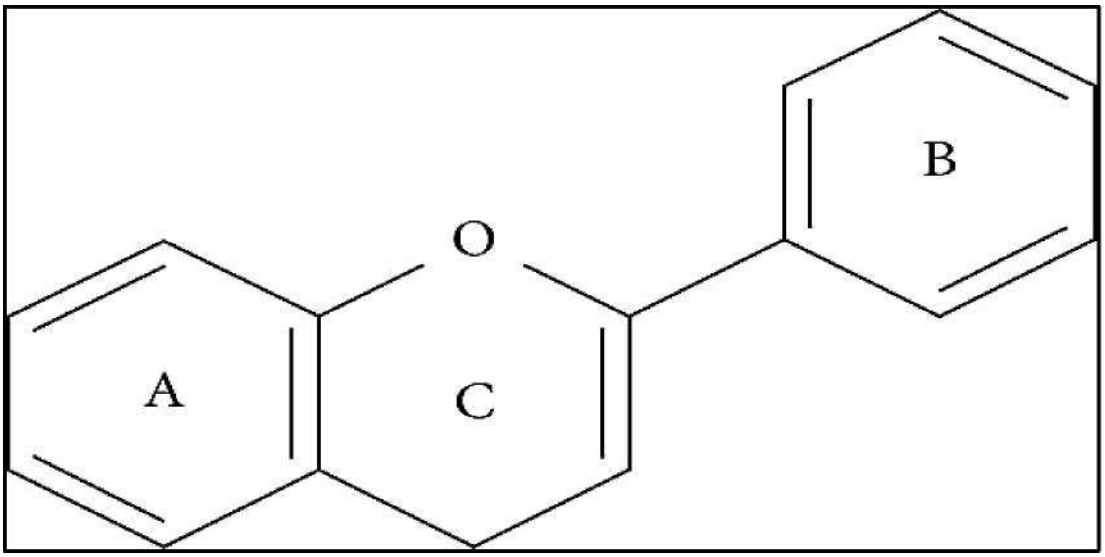

Figure 2.1.1 The basic skeleton structure of flavonoids, Diphenylpropane (C6-C3-C6) (Chagas et al., 2022).

The variations in its chemical profile can be detected based on geographical location, local floral diversity and seasonality (Kartal et al., 2002). This variability is reflected in the presence of specific phytochemicals composition in the propolis. For instance, propolis from tropical ecosystems, specifically from stingless bees might contain flavonoids or terpenoids that are derived from local flora plant species (Bankova et al., 2014). Whereas, in temperate regions propolis is often rich in cinnamic acid derivatives such as caffeic acid and its esters (Bans, et al., 2024). This variability further emphasizes the in-depth urge for chemical characterization in potential of therapeutic applications (Ban§ et al., 2024).

2.2 Propolis

Propolis is a resinous substance collected by honeybees from plant resins, buds, and exudates, which is then mixed with beeswax and enzymes to form a protective barrier within the hive (Anjum et al., 2019). This natural material serves multiple functions, including sealing cracks, preventing microbial contamination, and providing structural stability to the hive (Wagh, 2013) Typically, raw propolis consists of approximately 50% resinous compounds, 30% wax, 10% essential oils, 5% pollen, and 5% minor bioactive components, including amino acids, vitamins, and minerals (Syed Salleh et al., 2021). Figure 2.2 illustrates the raw state of propolis being ground into a powder.

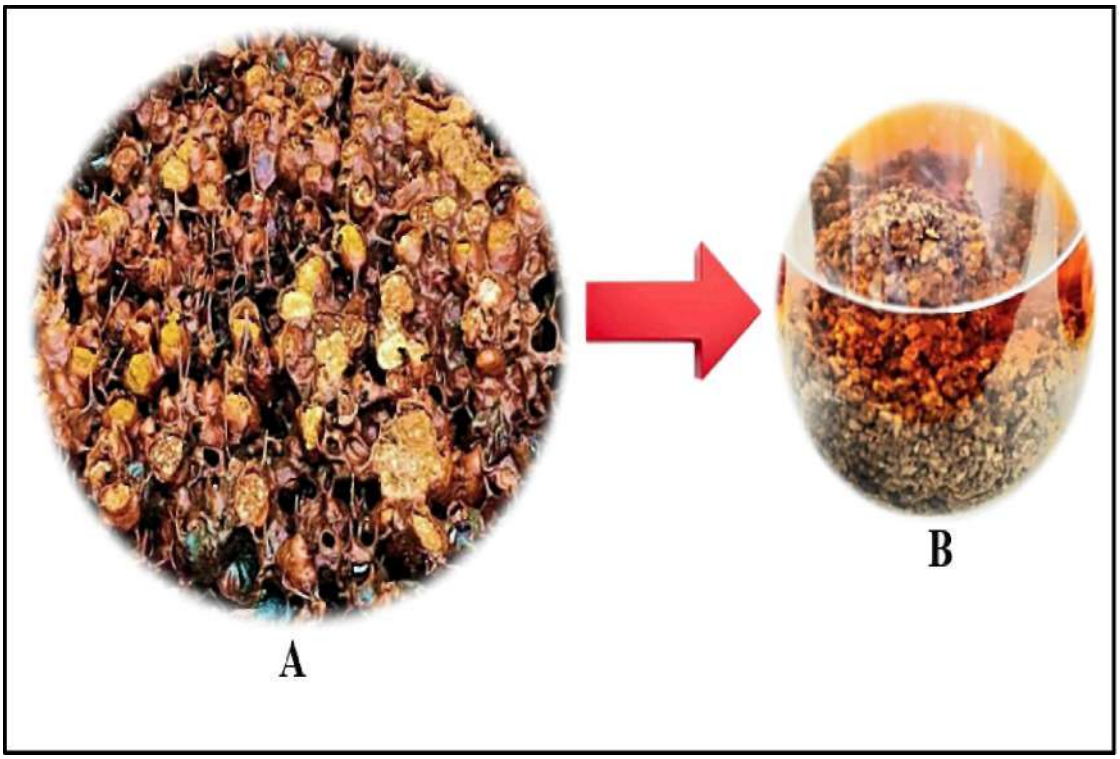

Figure 2.2 Stingless bee propolis samples. (A) Raw propolis. (B) Powder propolis.

The resin holds a diverse range of polyphenolic compounds, such as flavonoids, phenolic acids, and their esters, which contribute to its potent bioactivity (Syed Salleh et al., 2021). Terpenoids, coumarins, and lignans are also often detected, adding to its broad-spectrum pharmacological potential (Burdock, 1998). The antimicrobial properties of propolis contribute significantly to the overall health of the colony by preventing infections from bacteria, fungi, and viruses (Wagh, 2013). Additionally, propolis exhibits diverse bioactivities, including antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, immunomodulatory, and wound-healing effects, making it a valuable natural product for biomedical applications (Sforcin, 2016). However, raw propolis is unsuitable for direct application due to its complex matrix, which includes inert materials such as beeswax, environmental debris, and various contaminants that may dilute or interfere with its biological efficacy (Galeotti et al., 2018). Consequently, efficient extraction is required to isolate and concentrate the bioactive constituents. Ethanol remains the solvent of choice due to its high efficiency in recovering polyphenols while facilitating wax removal through selective solubility (Zhu et al., 2025).

The effectiveness of ethanol lies in its polar nature, which allows it to form hydrogen bonds with the hydroxyl (-OH) groups prevalent in polyphenol structures, thereby ensuring high extraction yields (Mohd Zain et al., 2025). In contrast, beeswax composed primarily of long-chain hydrocarbon esters and fatty acids, is highly non-polar. These lipid components exhibit negligible solubility in ethanol, particularly at lower temperatures, causing them to precipitate and be physically removed via filtration (Holser, 2009). While alternative solvents such as methanol, acetone, and ethyl acetate have been explored, ethanolic extracts of propolis (EEP) consistently demonstrate superior antioxidant and antimicrobial profiles, making them the standard for both research and commercial applications (Devequi Nunes et al., 2018; Yildinm, 2022).

The clinical applications of propolis have been extensively investigated across various biomedical fields, particularly in conditions driven by oxidative stress and chronic inflammation (da Silva et al., 2006). Its antioxidant activity plays a crucial role in combating oxidative stress, which is associated in numerous chronic diseases, including cardiovascular disorders, neurodegenerative conditions, and metabolic syndromes. The phenolic constituents of propolis neutralize free radicals and enhance the activity of endogenous antioxidant enzymes, thereby protecting cellular structures from oxidative damage. Furthermore, its anti-inflammatory properties are mediated through the inhibition of pro-inflammatory cytokines and the modulation of signalling pathways such as NF-KB and MAPK (da Silva et al., 2006).

One of the most promising applications of propolis is in wound healing, where its antimicrobial, anti-inflammatory, and regenerative properties accelerate tissue repair. Propolis has been shown to promote fibroblast proliferation, collagen synthesis, and angiogenesis, leading to improved wound closure and reduced scar formation. This makes it a valuable candidate for the development of advanced wound dressings and topical formulations (da Silva et al., 2006). Recent advancements in nanotechnology have further enhanced the bioavailability and therapeutic efficacy of propolis. Nanocarrier systems, such as liposomes, nanoparticles, and nanoemulsions, have been utilized to improve the solubility, stability, and controlled release of propolis-derived compounds. These formulations have demonstrated improved pharmacokinetics and

enhanced cellular uptake, making them promising for drug delivery applications (da Silva et al., 2006).

2.2.1 Polyphenol Compounds in Propolis Extracts

The bioactivity of propolis is largely attributed to its rich polyphenol content, which exhibits potent antioxidant, anti-inflammatory, and antimicrobial properties (Kitamura et al., 2019). These polyphenolic compounds play a crucial role in scavenging free radicals, modulating inflammatory pathways, and inhibiting microbial growth. The composition of polyphenols in propolis is influenced by various factors, including geographical location, seasonal changes, and floral diversity (Syed Salleh et al., 2021). Due to these variations, propolis samples are often analyzed for specific marker compounds to determine their chemical characteristics and potential biological effects (Wagh, 2013).

Approximately 500 compounds have been identified in propolis from different regions, with over 300 belonging to the polyphenol and terpene classes (Huang et al., 2014). For analytical characterization, techniques like GC-MS are particularly effective for profiling the volatile and semi-volatile fraction to identify these components (Huang et al., 2014). These include phenolic acids (such as caffeic acid, gallic acid, and p-coumaric acid), flavonoids (such as chrysin, galangin, quercetin, kaempferol, and pinocembrin), and lignans (Siripatrawan et al., 2013; Huang et al., 2014). Among these, flavonoids and phenolic acids are the predominant bioactive compounds responsible for propolis' strong antioxidant properties (Luna-Guevara et al., 2018). This includes terpenes, which are a major class whose have hydrocarbon skeletons and functional groups that has a characteristic pattern for GC-MS identification (Lee et al., 2023)

The primary compounds identified in propolis from non-tropical regions, such as Europe and North America, are flavonoids and phenolic acids, particularly caffeic acid and its derivatives (Maciejewicz et al., 2001). In contrast, Malaysian propolis has been reported to contain a unique profile, dominated by phenolic acids and terpenoids (Ismail et al., 2018; Nazir et al., 2018). A study by Syed Salleh et al. (2021) specifically identified caffeic acid, p-coumaric acid, naringenin, kaempferol, and quercetin as the key polyphenolic compounds in Malaysian *G. thoracica* propolis. These findings

highlight the distinct chemical composition of propolis derived from different ecological environments, emphasizing the importance of regional variations in determining its pharmacological potential.

Phenolic compounds and flavonoids are extensively studied for their antioxidant activity, which plays a crucial role in neutralizing free radicals and reducing oxidative stress (Tungmunnithum et al., 2018). Oxidative stress is a key factor in various chronic diseases, including cardiovascular disorders, neurodegenerative diseases, and cancer (Reddy, 2023). Studies have shown that phenolic compounds in propolis can significantly reduce lipid peroxidation, protect DNA from oxidative damage, and modulate key enzymes involved in cellular redox balance. Moreover, flavonoids like quercetin and kaempferol and Myricetin have been reported to exert anti-apoptotic and anti- inflammatory effects, further contributing to the therapeutic potential of propolis (Chagas et al., 2022) (Figure 2.2.1). Flavonoids, in particular, are known to enhance immune function, protect against oxidative stress, and exert neuroprotective effects (Jomova et al., 2025).

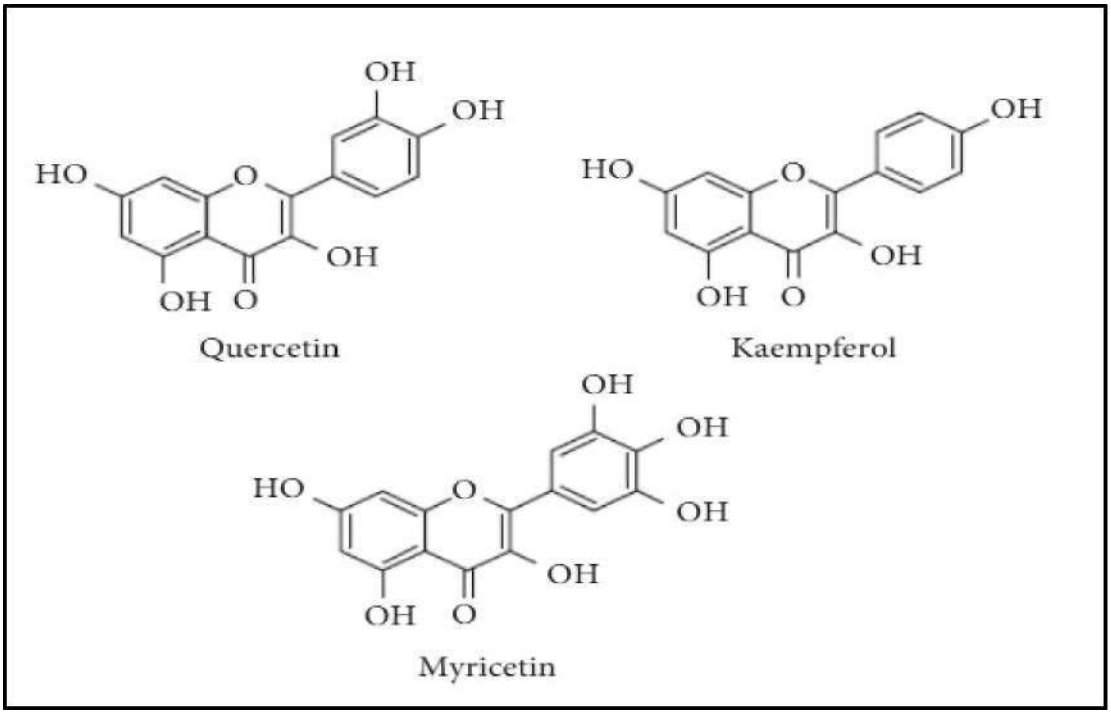

Figure 2.2.1 The chemical skeleton structures of quercetin, myricetin, and kaempferol (Chagas et al., 2022).

In addition to their antioxidant properties, polyphenols in propolis exhibit potent antimicrobial activity against a wide range of bacterial and fungal pathogens. The mechanism of action involves disruption of microbial cell membranes, inhibition of bacterial enzyme activity, and interference with quorum sensing pathways (Wagh, 2013). This antimicrobial activity has been particularly useful in dental applications, where propolis extracts have been incorporated into mouth rinses, toothpaste formulations, and wound-healing gels to combat oral infections and promote tissue regeneration.

The polyphenols composition of propolis also contributes to its anti-inflammatory properties. Phenolic acids, such as caffeic acid phenethyl ester (CAPE), have been shown to inhibit the activation of nuclear factor-kappa B (NF-KB), a key regulator of inflammation (Tungmunnithum et al., 2018). By modulating inflammatory pathways, propolis-derived polyphenols play a vital role in managing inflammatory diseases such as arthritis, asthma, and gastrointestinal disorders. Recent research has also explored the role of polyphenols in metabolic regulation, particularly in obesity and diabetes, where they enhance insulin sensitivity and inhibit adipogenesis.

2.2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis for Phytochemical Screening

Gas chromatography-mass spectrometry (GC-MS) is widely used as an analytical technique for identifying and quantifying phytochemicals in natural products. This method combines the separation power of gas chromatography (GC) with the structural elucidation capabilities of mass spectrometry (MS), making it particularly useful for analyzing volatile and semi-volatile organic compounds (Benkeblia, 2023). The primary advantage of GC-MS lies in its high sensitivity, reproducibility, and ability to provide comprehensive chemical profiles of complex mixtures such as plant extracts and essential oils (Filatov et al., 2023).

GC-MS operates in two distinct phases (Benkeblia, 2023). Separation by GC and detection by MS. The GC component utilizes an inert carrier gas, commonly helium or nitrogen, to transport the sample through a capillary column coated with a stationary phase. This allows for the separation of individual compounds based on their volatility

and polarity, with each analyte eluting at a characteristic retention time (Filatov et al., 2023). The separated compounds then enter the MS system, where they undergo ionization, typically through electron ionization (EI) or chemical ionization (CI). The resulting ionized molecules fragment into distinct patterns, which are recorded as mass spectra and compared against established spectral libraries such as NIST or Wiley for identification (Filatov et al., 2023).

In phytochemical research, GC-MS is instrumental in detecting bioactive constituents such as flavonoids, terpenoids, alkaloids, and polyphenols (Nazir et al., 2018). These compounds play crucial roles in antioxidant, antimicrobial, and anti-inflammatory activities, thereby contributing to the pharmacological potential of medicinal plants. For example, studies on propolis extracts have shown the presence of key bioactive compounds, including pinocembrin, chrysin, galangin, and caffeic acid phenethyl ester (CAPE), which exhibit strong antioxidant and antimicrobial properties (Nazir etal., 2018).

The applications of GC-MS in natural product research extend to standardization, ensuring batch-to-batch consistency in herbal medicines (Nazir et al., 2018). The ability to obtain detailed phytochemical profiles allows researchers to assess geographical variations in plant constituents, aiding in chemotaxonomic classification (Zhang et al., 2018). Sample preparation plays a critical role in the accuracy and reliability of GC-MS analysis (Nazir et al., 2018). Common techniques include solvent extraction using hexane, methanol, ethanol, or ethyl acetate for volatile essential oils (Zhang et al., 2018). Some phytochemicals require derivatization, such as silylation, to enhance their volatility and detectability in GC-MS.

The selection of extraction methods and derivatization procedures ensures optimal identification of target compounds (Moldoveanu & David, 2018). For non-volatile phytochemicals, such as phenolic acids, sugars, and amino acids, derivatization is a crucial preparation treatment step (Moldoveanu & David, 2018). The primary aims of derivatization are to increase analyte volatility by masking polar functional groups including -COOH, -OH, and -NFL and to improve thermal stability in order to prevent decomposition in the heated GC inlet, and to enhance detectability by forming derivatives with more favorable mass spectrometric patterns (Benkeblia, 2023).

The silylation using BSTFA or MSTFA, is a common method, which is replaces an active hydrogen with trimethylsilyl groups, making it a polar compound that sufficiently volatile and stable enough for GC-MS analysis (Shareef et al., 2006). The selection of extraction methods and derivatization procedures ensures optimal identification of target compounds (Benkeblia, 2023). Despite its established role as a foundation in phytochemical research, GC-MS has inherent limitations. A primary limitation is its inability to directly analyze non-volatile compounds, which require extensive sample preparation and prior derivatization (Shareef et al., 2006).

Furthermore, the technique can be costly due to demanding equipment maintenance and operational procedures (Benkeblia, 2023). Nevertheless, these challenges are being addressed through methodological advancements. The development of hyphenated techniques, such as the coupling of GC-MS with liquid chromatography (LC-MS) or nuclear magnetic resonance (NMR), has significantly expanded its analytical scope. Consequently, GC-MS continues to provide a powerful and evolving platform for comprehensive natural product characterization, quality control, and bioactivity assessment.

2.3 Structure And Functions of Adipose Tissue

Adipose tissue, commonly referred to as adipocytes, is a specialized connective tissue that plays a crucial role in energy homeostasis, metabolic regulation, and endocrine signalling (Coelho et al., 2018). It is widely recognized as a dynamic endocrine organ that not only stores and releases energy but also secretes numerous bioactive molecules, including hormones and cytokines, that influence various physiological processes (Coelho et al., 2018). Functioning as a key integrator of energy balance, adipose tissue is involved in lipid metabolism, thermoregulation, and immune modulation. It also plays a significant role in glucose homeostasis, insulin sensitivity, and inflammatory responses, making it a vital part of the body's metabolic network (Zhai et al., 2020).

Adipocytes are derived from multipotent mesenchymal stem cells (MSCs), particularly those originating from bone marrow mesenchymal stem cells (BMSCs) (Zhai et al., 2020). These precursor cells undergo differentiation and maturation through

a complex series of molecular and cellular events regulated by various transcription factors, including peroxisome proliferator-activated receptor gamma (PPAR-y) and CCAAT/enhancer-binding proteins (C/EBPs) (Zhai et al., 2020). The differentiation process leads to the formation of mature adipocytes, which constitute the predominant cellular component of adipose tissue (Niemela et al., 2008). In addition to mature adipocytes, adipose tissue comprises several other cell types, collectively known as stromal-vascular cells (SVCs) (Coelho et al., 2018). These include fibroblasts, pericytes, endothelial cells, immune cells such as macrophages, and adipogenic progenitor cells, all of which contribute to the overall functionality and homeostasis of adipose tissue (Coelho et al., 2018).

Adipose tissue is broadly classified into two major subtypes: white adipose tissue (WAT) and brown adipose tissue (BAT), each exhibiting distinct structural and functional characteristics (Niemela et al., 2008). White adipose tissue is the predominant form of fat storage in the human body and is widely distributed across various anatomical regions, including the subcutaneous and visceral compartments (Coelho et al., 2018). Its primary function is to serve as an energy reservoir by storing excess energy in the form of triacylglycerol. During periods of energy deficiency, WAT releases stored lipids as free fatty acids, which serve as an essential energy source for peripheral tissues (Niemela et al., 2008).

On the other hand, brown adipose tissue (BAT) is relatively scarce and primarily found in specific anatomical locations such as the interscapular, perirenal, and supraclavicular regions (Wu et al., 2013). Unlike WAT, which primarily stores energy, BAT is highly specialized for thermogenesis, a process that generates heat through the oxidation of fatty acids. This thermogenic capacity is largely attributed to the presence of uncoupling protein 1 (UCPl), a mitochondrial protein that enables the dissipation of energy as heat rather than ATP production (Wu et al., 2013). The presence of UCPl-rich mitochondria gives BAT its characteristic brown colour. BAT plays a crucial role in regulating body temperature, particularly in neonates and individuals exposed to cold environments (Wu et al., 2013). The functional distinction between WAT and BAT highlights their complementary roles in supporting metabolic balance and body weight regulation (Zhai et al., 2020).

Beyond its role in energy storage and thermogenesis, white adipose tissue is also recognized as an active endocrine organ that secretes a wide array of adipokines hormones and signalling molecules that mediate metabolic and inflammatory processes (Zhai et al., 2020). Among the most well-characterized adipokines is leptin, a hormone predominantly produced by adipocytes. Leptin plays a central role in appetite regulation, energy expenditure, and body weight homeostasis (Vazquez-Vela et al., 2008). It exerts its physiological effects by binding to leptin receptors in the hypothalamus, where it modulates neuroendocrine pathways to suppress food intake and enhance energy expenditure (Pico et al., 2022). Consequently, leptin is often referred to as an anti- obesity hormone due to its pivotal role in reducing appetite and promoting weight loss.

In conclusion, adipose tissue is a multifaceted organ that extends far beyond its traditional role as a passive fat storage depot. It actively participates in endocrine regulation, immune function, and metabolic homeostasis. The intricate interplay between WAT and BAT, along with the diverse array of adipokines secreted by adipocytes, underscores the critical importance of adipose tissue in human physiology. Ongoing research continues to unravel new insights into the regulatory mechanisms governing adipose tissue function, offering potential therapeutic targets for obesity-related metabolic disorders and other chronic diseases.

2.3.1 *Adipogenesis*

Adipogenesis is a complex and tightly regulated biological process through which preadipocytes differentiate into mature adipocytes, leading to the formation of adipose tissue in various parts of the body (Zhai et al., 2020). This process is essential for maintaining energy homeostasis, as adipocytes serve as the primary storage site for excess energy in the form of triglycerides. Adipogenesis plays a fundamental role in lipid metabolism and energy balance, making it a critical factor in both normal physiological functions and pathological conditions such as obesity (Ju et al., 2011; Haider & Larose, 2019).

The dysregulation of adipogenesis has been implicated in metabolic disorders, including insulin resistance, type 2 diabetes, and cardiovascular diseases, highlighting its significance in human health. Adipogenesis occurs in two distinct phases, hyperplasia and hypertrophy (Figure 2.4.1). Hyperplasia refers to an increase in the number of adipocytes through the proliferation and differentiation of preadipocytes. This phase is particularly prominent during early development and puberty, when new adipocytes are generated to accommodate increased energy storage needs. Hypertrophy, on the other hand, is characterized by the enlargement of existing adipocytes as they accumulate lipids. While both hyperplasia and hypertrophy contribute to adipose tissue expansion, hypertrophy is more commonly associated with obesity-related complications (Ju et al., 2011; Haider & Larose, 2019). The excessive accumulation of lipids in adipocytes leads to cellular dysfunction, increased inflammation, and the development of insulin resistance, which are hallmarks of obesity and metabolic syndrome.

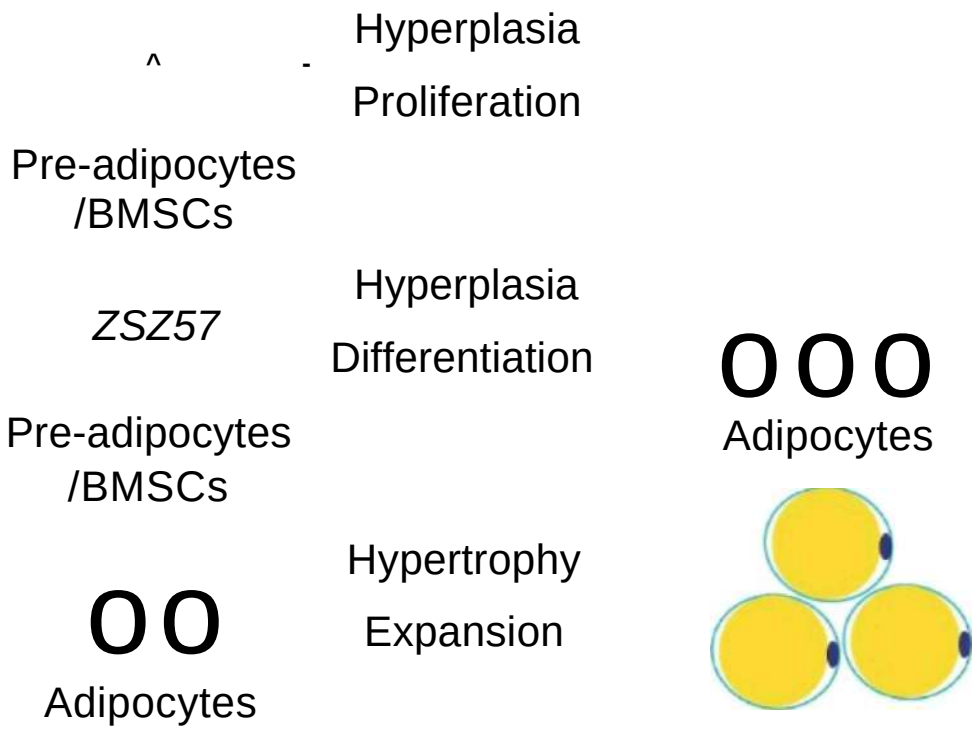

Figure 2.3.1 The schematic diagram of the process of adipogenesis (Zhai et al., 2020).

At the molecular level, adipogenesis is regulated by a sophisticated network of transcription factors and signalling pathways that coordinate the differentiation of preadipocytes into mature adipocytes. The key adipogenic transcription factors include peroxisome proliferator-activated receptor gamma (PPARy), CCAAT-enhancer-binding protein beta (C/EBPP), and CCAAT-enhancer-binding protein alpha (C/EBPa), which function in a sequential and synergistic manner to drive adipocyte differentiation (Niemela et al., 2008). PPARy is recognized as the master regulator of adipogenesis, playing a crucial role in initiating and maintaining the differentiated state of adipocytes (Siersbaek et al., 2010). This nuclear receptor facilitates the expression of genes involved in lipid metabolism, insulin sensitivity, and adipocyte-specific functions. C/EBPP acts upstream of PPARy, inducing its expression during the early stages of adipogenesis, while C/EBPa collaborates with PPARy to sustain adipocyte differentiation and function (Nielsen et al., 2008).

The regulation of adipogenesis involves multiple signalling pathways, including the insulin signalling pathway, Wnt/p-catenin pathway, and mitogen-activated protein kinase (MAPK) pathway. The insulin signalling pathway plays a fundamental role in adipogenesis by promoting glucose uptake and lipid accumulation in adipocytes. Insulin activates phosphoinositide 3-kinase (PI3K) and Akt, leading to the upregulation of PPARy and C/EBPa expression, thereby enhancing adipocyte differentiation. Conversely, the Wnt/p-catenin signalling pathway acts as a negative regulator of adipogenesis. When activated, Wnt/p-catenin signalling inhibits the expression of PPARy and C/EBPa, thereby preventing adipocyte differentiation and promoting mesenchymal stem cell differentiation into osteoblasts instead of adipocytes (Xiao et al., 2010).

In recent years, the role of oxidative stress in adipogenesis has gained significant attention. Reactive oxygen species (ROS) are highly reactive molecules that are generated as byproducts of cellular metabolism and play a critical role in cell signalling and homeostasis under normal physiological conditions. However, excessive ROS production resulting from oxidative stress can lead to cellular damage and metabolic dysfunction, contributing to obesity and related disorders (Valko et al., 2006; Furst, 2009). ROS influence adipogenesis by modulating the activity of key transcription factors and signalling pathways. Studies have demonstrated that elevated ROS levels

promote adipocyte differentiation and lipid accumulation, exacerbating adipose tissue expansion and obesity-related complications (Masschelin et al., 2020).

During adipogenesis, preadipocytes exhibit increased expression of mRNAs encoding key adipogenic transcription factors, including PPARy, C/EBPP, and C/EBPa. These transcription factors function to upregulate the expression of genes that define the adipocyte phenotype and activate pathways required for terminal differentiation (Niemela et al., 2008). PPARy is particularly important for maintaining the differentiated state of adipocytes, ensuring their proper function and lipid storage capacity (Siersbaek et al., 2010). C/EBPa, in conjunction with PPARy, plays a vital role in establishing the characteristics of mature adipocytes by regulating genes involved in lipid metabolism, insulin sensitivity, and adipokine secretion (Nielsen et al., 2008). Notably, both PPARy and C/EBPa are considered master adipogenic transcription factors that drive the formation and expansion of adipose tissue. Conversely, inhibiting PPARy and C/EBPa has been shown to suppress adipocyte differentiation and reduce fat accumulation, highlighting their potential as therapeutic targets for obesity treatment (Xiao etal., 2010).

In addition, oxidative stress has been shown to impair mitochondrial function in adipocytes, leading to metabolic disturbances and insulin resistance. Understanding the interplay between oxidative stress, inflammation, and adipogenesis may provide novel insights into the development of anti-obesity interventions targeting adipose tissue dynamics and metabolic health. In conclusion, adipogenesis is a highly coordinated process involving cellular differentiation, transcriptional regulation, and metabolic adaptation. The balance between adipocyte hyperplasia and hypertrophy determines adipose tissue expansion and influences metabolic homeostasis. The intricate regulatory network of transcription factors, signalling pathways, and oxidative stress responses underscores the complexity of adipogenesis and its role in obesity and related metabolic disorders.

2.3.2 Antioxidants, Oxidative Stress and Reactive Oxidative Species

A free radical is defined as a highly reactive and unstable molecular species distinguished by the presence of an unpaired electron located in its outermost electron

orbital. This unpaired electron renders free radicals dramatically reactive within biological systems, compelling them to engage in rapid chemical reactions (Ratnam et al., 2006). These reactive molecules possess the ability to act as either oxidants or reductants based on their role in the electron exchange process, by donating or accepting electrons, respectively. Through these actions, free radicals contribute significantly to various oxidative processes within the body and are continuously generated as a natural byproduct of aerobic metabolism. They play crucial roles in an array of cellular processes, including energy production, immune defense mechanisms, and vital cell signalling pathways (Ratnam et al., 2006). Nonetheless, when there is an excessive accumulation of free radicals, it can lead to substantial cellular damage and adversely contribute to the development of various pathological conditions, as illustrated in Figure 2.3.2.

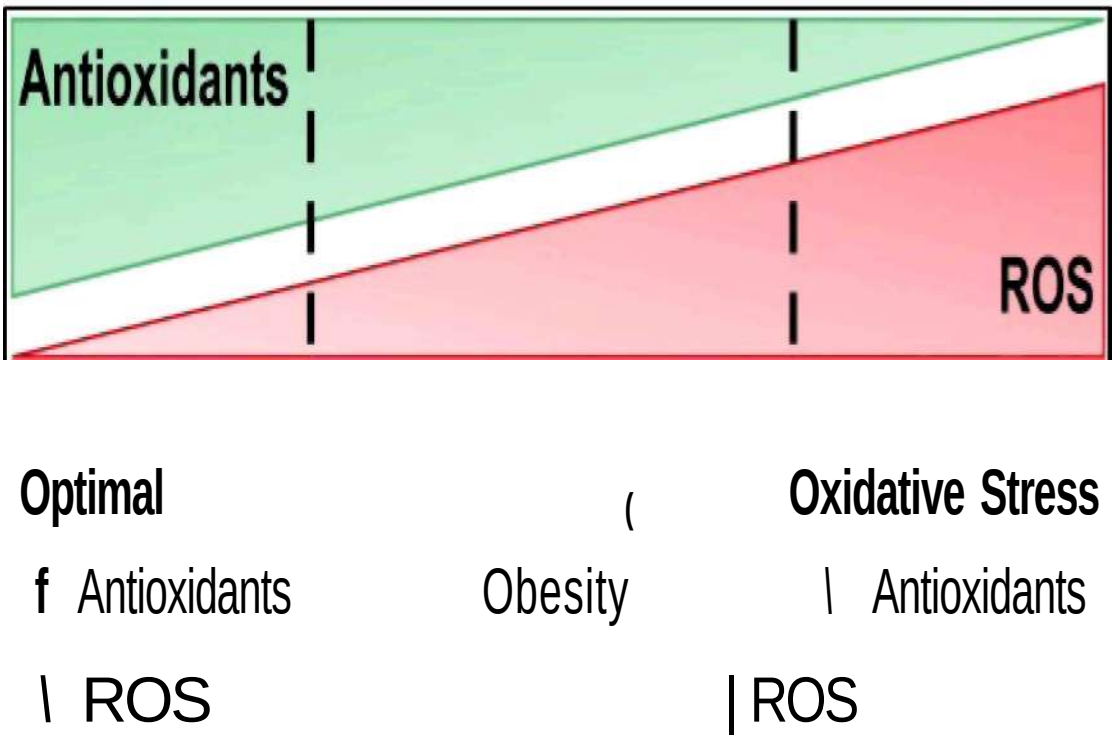

Figure 2.3.2 The stability of antioxidants and ROS control oxidative stress (Masschelin et al., 2020).

Reactive oxygen species (ROS) represent a subclass of chemically reactive free radicals that specifically contain oxygen within their molecular structure (Valko et al., 2007). Among the most commonly encountered ROS are hydrogen peroxide (H2O2), hydroxyl radicals (*OH), and superoxide anions (O2-). These species are primarily

generated during mitochondrial oxidative phosphorylation and through numerous enzymatic reactions that occur within the cell (Halliwell, 1995; Valko et al., 2007). While ROS are essential for several physiological functions, including roles in immune defense and redox signalling. Overproduction can exceed the body's inherent antioxidant defense capabilities, leading to conditions of oxidative stress.

Oxidative stress is characterized as a state where the generation of ROS surpasses the cellular antioxidant capacity, resulting in an imbalance that instigates oxidative damage to critical cellular components such as lipids, proteins, and DNA (Halliwell, 1995; Valko et al., 2007). This imbalance contributes notably to cellular dysfunction. Various external environmental factors, including ultraviolet (UV) radiation, air pollution, tobacco smoke and exposure to ionizing radiation serve to exacerbate the accumulation of ROS and contribute to the onset of oxidative stress (Furst, 2009). In the context of obesity, oxidative stress is particularly significant; excessive accumulation of lipids can increase mitochondrial ROS production, which in turn triggers systemic inflammation, insulin resistance, and a range of metabolic dysfunctions (Valko et al., 2006; Furst, 2009). Prolonged exposure of cells to conditions of oxidative stress can precipitate chronic diseases including cardiovascular disorders, neurodegenerative diseases, diabetes, and various forms of cancer due to the induction of genetic mutations and cellular apoptosis (Masschelin et al., 2020).

Antioxidants are pivotal for counteracting oxidative stress as they work by scavenging free radicals and neutralizing ROS, thereby preventing cellular damage that can arise from oxidative stress. These protective molecules function through diverse mechanisms such as donating electrons to stabilize reactive free radicals, chelating metal ions that serve as catalysts in oxidative reactions and enhancing the activity of endogenous antioxidant enzymes (DeFeudis et al., 2003). Antioxidants can be categorized based on their characteristics into two main groups. Enzymatic antioxidants, which include superoxide dismutase, catalase, and glutathione peroxidase, and non- enzymatic antioxidants, which encompass a variety of compounds such as vitamin C, vitamin E, flavonoids, polyphenols, and carotenoids (Kumar et al., 2022). The optimal cellular redox balance is achieved when antioxidant levels are sufficiently elevated to effectively neutralize excessive ROS, ensuring proper cellular function and offering a protective mechanism against the diseases that can arise from oxidative stress

(Masschelin et al., 2020). By facilitating this balance, antioxidants play a crucial role in maintaining overall health and preventing various chronic conditions.

2.4 **Antioxidant and Anti-Adipogenic** Assays

2.4.1 **Diphenyl-1-picrylhydrazyl (DPPH)** assay

The Diphenyl-1-picrylhydrazyl (DPPH) assay is one of the most employed methods for evaluating the antioxidant potential of natural compounds, particularly in plant extracts and other biologically active substances. This method is based on the principle of electron transfer, where the stable DPPH radical, which has a deep violet colour in solution, is reduced to a yellow-coloured diphenylpicrylhydrazine upon reaction with an antioxidant compound (Nafi et al., 2019). This colour change can be quantitatively measured using a spectrophotometer at a wavelength of 517 nm, providing a direct indication of the free radical scavenging ability of the tested sample (Shahidi & Zhong, 2015).

The Diphenyl-1-picrylhydrazyl (DPPH) assay is one of the most employed methods for evaluating the antioxidant potential of natural compounds, particularly in plant extracts and other biologically active substances. This method is based on the principle of electron transfer, where the stable DPPH radical, which has a deep violet colour in solution, is reduced to a yellow-coloured diphenylpicrylhydrazine upon reaction with an antioxidant compound (Nafi et al., 2019). This colour change can be quantitatively measured using a spectrophotometer at a wavelength of 517 nm, providing a direct indication of the free radical scavenging ability of the tested sample (Shahidi & Zhong, 2015).

Various studies have utilized the DPPH assay to assess the free radical scavenging properties of propolis extracts, often correlating antioxidant activity with the presence of bioactive compounds such as flavonoids, polyphenols, and other secondary metabolites. The strong antioxidant potential of propolis has been linked to its ability to neutralize oxidative stress, which is associated with various diseases, including cardiovascular disorders, neurodegenerative conditions, and aging-related

damage. By quantifying the scavenging ability of propolis-derived extracts, researchers can better understand their potential therapeutic applications.

2.4.2 Ferric Reducing Antioxidant Power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay is another widely used technique for assessing the antioxidant capacity of natural extracts. Unlike the DPPH assay, which measures free radical scavenging activity, the FRAP assay focuses on the reducing power of a sample (Munteanu & Apetre, 2021). This method is based on the ability of antioxidants to reduce ferric (Fe^{3}+) to ferrous (Fe^{2}+) ions in the presence of the tripyridyltriazine (TPTZ) reagent, resulting in the formation of a blue-coloured Fe^{2}+TPTZ complex. The intensity of this colour change is measured spectrophotometrically at a wavelength of 593 nm and is directly proportional to the antioxidant potential of the sample. The FRAP assay is particularly useful in quantifying the total antioxidant power of a sample, as it does not rely on radical scavenging but rather evaluates the electron- donating capacity of antioxidants.

This method is advantageous due to its high sensitivity, specificity, and rapid execution time. Unlike other assays that may be influenced by reaction kinetics, the FRAP assay provides a stable and consistent measure of reducing power (Zarate et al., 2018). In the context of natural product research, including studies on propolis, the FRAP assay has been extensively applied to determine the total antioxidant potential of various extracts. Propolis, a resinous substance produced by bees, is known to contain high concentrations of flavonoids, phenolics, and other bioactive compounds with strong reducing properties (Kurek-Gorecka et al., 2022). The antioxidant potential measured using the FRAP assay has been correlated with the ability of propolis to protect against oxidative damage, reduce inflammation, and support cellular health. By employing the FRAP assay alongside other antioxidant assays, researchers can obtain a more comprehensive understanding of the antioxidant mechanisms of propolis-derived compounds.

2.4.3 3T3-L1 Preadipocytes as an *In Vitro* Model for Evaluating Anti- Adipogenic Activity

Upon stimulation, 3T3-L1 preadipocytes undergo a series of morphological and biochemical changes that lead to the formation of lipid-laden adipocytes (Ji et al., 2015). This process is regulated by key adipogenic transcription factors, including peroxisome proliferator- activated receptor gamma (PPARy), CCAAT/enhancer-binding protein alpha (C/EBPa), and fatty acid-binding protein 4 (FABP4). These factors play a critical role in the regulation of adipogenesis, and their expression levels are commonly used as markers of adipocyte differentiation (Ji et al., 2015). In studies of natural products, including propolis extracts, 3T3-L1 cells serve as a valuable tool to assess anti-adipogenic properties. The ability of propolis-derived compounds to inhibit adipogenesis is often evaluated through various techniques, including Oil Red O staining, which allows visualization and quantification of intracellular lipid accumulation (Xiao et al., 2010).

Additionally, molecular analyses such as RT-qPCR and Western blotting are used to examine the expression levels of key adipogenic genes and proteins, providing insights into the mechanisms by which propolis influences fat metabolism (Ji et al., 2015). Several studies have reported that propolis extracts exhibit significant anti-adipogenic effects by modulating adipocyte differentiation and lipid accumulation (Nielsen et al., 2008). These effects are primarily attributed to the presence of flavonoids, polyphenols, and other bioactive compounds that interfere with the signalling pathways involved in adipogenesis (Nielsen et al., 2008). By inhibiting PPARy activation and downregulating adipogenic gene expression, propolis-derived compounds have shown potential in preventing excessive fat accumulation and obesity-related metabolic disorders (Xiao et al., 2010). Overall, the use of the 3T3-L1 cell model in anti-adipogenic research provides valuable insights into the potential of natural compounds to regulate adipogenesis and lipid metabolism. The findings from such studies contribute to the growing body of evidence supporting the role of propolis as a functional food ingredient with potential applications in obesity management and metabolic health.

2.4.4 Quantitative Real-Time PCR (qRT-PCR) Analysis in Gene Expression and Anti-Adipogenic Activity

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) is a highly sensitive and widely utilized molecular technique for analyzing gene expression levels in biological samples (Gustafson & Smith, 2006). It has revolutionized gene expression studies by providing precise quantification of mRNA transcripts, a process at the molecular level. This technique is especially valuable in studies focusing on adipogenesis, obesity-related metabolic disorders, and the identification of potential anti-adipogenic agents (Gustafson & Smith, 2006). qRT-PCR enables accurate detection and quantification of key transcription factors and metabolic genes that regulate adipocyte differentiation, lipid metabolism, and inflammatory responses, offering critical insights into the underlying molecular mechanisms of adipogenesis (Farooqi et al., 1999).

One of the primary applications of qRT-PCR in adipogenesis research is the evaluation of adipogenic gene expression. Adipogenesis is a complex, multi-step process that involves the differentiation of preadipocytes into mature adipocytes. This differentiation is primarily regulated by key transcription factors, including peroxisome proliferator-activated receptor gamma (PPARy), CCAAT/enhancer-binding proteins (C/EBPa and C/EBPP), sterol regulatory element-binding proteins (SREBP-lc), and fatty acid-binding proteins (Farooqi et al., 1999). These factors coordinate the activation of lipogenic enzymes and lipid transport proteins essential for the development and function of adipocytes. Through qRT-PCR, researchers can quantitatively assess the expression of these pivotal genes, allowing for a deeper understanding of the molecular events driving adipogenesis (Heid et al., 1996).

In the context of anti-adipogenic activity, qRT-PCR serves as a crucial tool for evaluating the impact of various bioactive compounds, natural extracts, and pharmacological agents on adipogenesis Rayalam et al., 2008). The identification of natural products with anti-adipogenic properties has gained significant interest due to the rising global prevalence of obesity and its associated metabolic disorders. By analyzing gene expression patterns, researchers can determine the extent to which specific compounds inhibit adipogenic differentiation. For instance, plant-derived

polyphenols, flavonoids, and terpenoids have been shown to exert anti-adipogenic effects by downregulating key transcription factors such as PPARy and C/EBPa while simultaneously upregulating genes involved in lipid oxidation, mitochondrial biogenesis, and energy expenditure (Rayalam et al., 2008). This gene expression analysis provides substantial evidence supporting the anti-obesity potential of bioactive compounds derived from medicinal plants and functional foods.

The utility of qRT-PCR extends beyond simple gene expression analysis, as it also provides insights into epigenetic modifications that influence adipogenesis. Epigenetic regulation, including DNA methylation, histone modifications, and microRNA-mediated gene silencing, plays a crucial role in modulating adipogenic gene expression. qRT-PCR can be employed to quantify the expression levels of microRNAs (miRNAs) that target key adipogenic transcription factors (Gustafson & Smith, 2006). For instance, miR-27 has been reported to suppress PPARy expression, thereby inhibiting adipocyte differentiation, while miR-155 promotes inflammation in adipose tissue. Understanding these epigenetic mechanisms through qRT-PCR-based analysis opens new avenues for developing targeted anti-obesity therapies (Gustafson & Smith, 2006).

In conclusion, qRT-PCR is a powerful and versatile molecular tool for elucidating the mechanisms of adipogenesis and assessing the anti-adipogenic effects of various compounds. By providing accurate quantification of gene expression, this technique enhances our understanding of adipocyte differentiation, lipid metabolism, oxidative stress, and inflammation. The insights gained from qRT-PCR studies contribute to the development of effective anti-obesity interventions by identifying molecular targets for therapeutic strategies.

2.5 Effect of Propolis Extract on Adipogenesis

Propolis has garnered considerable scientific interest due to its diverse bioactive properties, including its potential role in adipogenesis and lipid metabolism (Rivera-Yanez et al. 2020). Multiple studies have provided compelling evidence that propolis exerts anti-adipogenic effects by modulating key molecular pathways governing adipocyte differentiation, lipid storage, and energy metabolism (Rivera-Yanez et al.

2020). These findings suggest that propolis and its bioactive constituents may serve as potential therapeutic agents for obesity management. The following discussion summarizes the impact of propolis on adipogenesis, with reference to relevant scientific studies (Table 2.5).

Table 2.5
The anti-adipogenic effects of Brazilian propolis extract (Rivera-Yanez et al. 2020).

Location	Propolis Type	Study Type		Effects on Obesity-related diseases	References
Brazil	Red Propolis	*In Vitro*	3T3-L1 Preadipocytes	Induces adiponectin mRNA through activation of the adiponectin promoter by PPAR-y.	Lio et al., 2010
Brazil	Propolis	*In Vivo*	HFD-induced obese Wistar rats	Repress weight gain of total white adipose tissues. Level of protein PPAR- y in adipose tissues was lower.	Ichi et al., 2009
Brazil	Green Propolis	*In Vitro*	3T3-L1 Preadipocytes	Elevates leptin expression.	Washioetal., 2015
Brazil	Propolis	*In Vivo*	HFD-induced obese C57BL/6N Mice	Reduces body weight gain and serum triglycerides. Regulates mRNA expression associated with fatty acid.	Koya-Miyata et al., 2009

Adipogenesis, the process by which preadipocytes differentiate into mature adipocytes, is tightly regulated by a network of transcription factors, including peroxisome proliferator-activated receptor gamma (PPAR-y) and CCAAT/enhancer-binding proteins (C/EBPs) (Rivera-Yanez et al. 2020). These transcription factors play pivotal roles in regulating the expression of genes involved in lipid accumulation, insulin sensitivity, and adipokine secretion (Rivera-Yanez et al. 2020). Several studies have demonstrated that bioactive compounds in propolis, such as flavonoids, phenolic acids, and terpenes, interact with these signalling pathways, influencing adipogenic outcomes Ichi et al. (2009). A study by Shin et al. (2014) reported that specific compounds can mediate anti-adipogenic effects include the phenolic ester caffeic acid phenethyl ester (CAPE), which can reduce mRNA expression including the PPAR-y expression. The flavonoids compound including quercetin and chrysin both are able to downregulate key markers adipogenic such as C/EBPa (Shin et al., 2014).

A study by Lio et al. (2010) demonstrated that treatment with Brazilian red propolis for three days significantly enhanced adiponectin mRNA expression in 3T3-Ll preadipocytes. This upregulation was attributed to the activation of the adiponectin promoter through the PPAR-y signalling pathway, suggesting that propolis may enhance insulin sensitivity and overall metabolic regulation. Adiponectin is a key adipokine involved in glucose homeostasis and lipid metabolism, indicating that propolis could play a role in mitigating metabolic dysfunctions associated with obesity and type 2 diabetes. Similarly, an in vivo study by Ichi et al. (2009) investigated the impact of propolis supplementation in high-fat diet (HFD)-induced obese Wistar rats. The results revealed that eight weeks of propolis administration led to a significant reduction in total white adipose tissue weight, coupled with decreased PPAR-y protein expression in adipose tissues. These findings suggest that propolis suppresses adipocyte differentiation and lipid accumulation, which could be beneficial in controlling obesity-related metabolic complications.

Further supporting these observations, Washio et al. (2015) conducted an *in vitro* study using differentiated 3T3-L1 adipocytes and reported that Brazilian green propolis directly upregulated leptin expression. Leptin is a crucial adipokine that regulates appetite and energy homeostasis, further suggesting that propolis may contribute to weight regulation and metabolic balance. By modulating leptin secretion, propolis could

influence satiety signals and energy expenditure, reducing the risk of excessive weight gain. In another study, Koya-Miyata et al. (2009) examined the effects of Brazilian propolis on body fat and lipid metabolism in HFD-induced obese C57BL/6N mice. Propolis was administered intragastrical twice daily for ten days, leading to notable reductions in body weight gain and serum triglyceride levels. These findings provide further evidence that propolis enhances lipid metabolism, thereby preventing excessive fat accumulation and reducing obesity-related risks.

Moreover, Kitamura et al. (2019) presented comprehensive evidence supporting the anti-adipogenic properties of propolis. Their research highlighted that propolis not only regulates adipogenesis but also influences food intake, adipokine secretion, and overall metabolic homeostasis. The proposed mechanisms underlying these effects involve modulation of inflammatory cytokine expression, inhibition of lipogenesis, and enhancement of mitochondrial function, collectively contributing to its potential in obesity management. Beyond its role in adipogenesis, propolis exhibits anti-inflammatory and antioxidant properties, which may further contribute to its metabolic benefits Kitamura et al. (2019). Chronic inflammation and oxidative stress are major contributors to obesity-related complications, including insulin resistance and cardiovascular disease. Propolis has been shown to downregulate pro-inflammatory cytokines such as tumour necrosis factor- alpha (TNF-a) and interleukin-6 (IL-6), which are known to disrupt insulin signalling and promote adipocyte dysfunction. Additionally, propolis-derived flavonoids and phenolic acids possess strong antioxidant activity, reducing oxidative stress and enhancing mitochondrial function in adipose tissues.

Collectively, these studies provide substantial evidence that propolis exhibits significant anti-adipogenic and lipid-lowering effects, making it a promising natural compound for obesity management and metabolic health. However, despite these promising findings, further research is necessary to fully elucidate the specific bioactive compounds responsible for these effects, their precise molecular mechanisms, and their potential therapeutic applications in clinical settings. Future studies should also explore the bioavailability, pharmacokinetics, and long-term safety of propolis-based interventions to establish its efficacy as a natural anti-obesity agent.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Propolis Collection

Propolis samples for this study were collected by our group in December 2022 from three different commercial apiaries located within the state of Selangor, Malaysia. The selection of location with varying surrounding vegetation was deliberate to assess the effect of floral origin and location variation on a chemical profile and bioactivities of the propolis extracts. The raw propolis was sourced from identified *G. thoracica* stingless bee hives located in Serdang (SER), coordinates N 2° 58' 45.84" E 101° 41' 51.72", an urban-agricultural area dominated by vegetation from the *Sapindaceae, Cucurbitaceae, Palmaceae, and Anacardiaceae* families. Second location located at ShahAlam (SA), coordinates N 3° 3'46.08"E 101° 32'2.4", a suburban landscape with flora primarily from the *Malvaceae, Rutaceae, Fabaceae, Combretaceae,* and *Sapindaceae* families and third location located at Hulu Bernam (HB), coordinates N 3° 40' 42.1818" E 101° 31' 14.5416", a semi-rural region characterized by a rich diversity of ornamental, fruit, and resin-secreting trees from families including *Myrtaceae, Fabaceae, Lamiaceae, Moraceae, Anacardiaceae,* and *Sapindaceae.* To ensure the accurate and reliability in this study, an entomologist from the Malaysian Agricultural Research and Development Institute (MARDI) in Serdang, Selangor, was consulted to verify the stingless bee species identification on which the propolis was sourced to producing the propolis. This identification process is crucial for establishing the connection between the bee species and the resulting propolis composition. For the purpose of this study, the collected propolis samples are henceforth referred to abbreviation, SER for Serdang, SA for Shah Alam, and HB for Hulu Bernam (Table 3.1).

Table 3.1
Description of propolis collection sites and environmental characteristics.

Location	Geographical Coordinates	Landscape Type	Dominant Vegetation (Family)	Key Floral Characteristics
SER	N 2° 58' 45.84" E 101° 41' 51.72"	Urban Agricultural	*Sapindaceae, Cucurbitaceae, Palmaceae (Arecaceae), Anacardiaceae*	Dominated by fruit trees (Mangoo, Lychee and Rambutan)
SA	N 3° 3'46.08" E 101° 32' 2.4"	Suburban	*Malvaceae, Rutaceae, Fabaceae, Combretaceae, Sapindaceae*	Mix of ornamental diversity, and floral garden (citrus)
HB	N3°40'42.1818" E 101° 31' 14.5416"	Semi-rural	*Myrtaceae, Fabaceae, Lamiaceae, Moraceae, Anacardiaceae, Sapindaceae*	Mix of ornamental, fruit, and resin-secreting trees (Melaleuca)

3.2 Preparation of Ethanolic Extract Propolis (EEP)

The preparation of the ethanolic extract of propolis (EEP) from the three locations was conducted following the methodology outlined by Pobiega et al. (2019) with modifications. Each sample of raw propolis from location at SER, SA, and HB was processed separately. Approximately, 10 grams of each crude, solid propolis sample was first finely ground separately into a powder using a sterile mortar and pestle. This process increased the surface area to facilitate efficient extraction. The powder from each location was then macerated separately in 100 mL of 70% (v/v) ethanol, with a ratio of 1:10 (w/v). Each mixture was placed on a shaker set at 250 rpm for continuous shake and maintained at a controlled room temperature of 25°C for 48 hours to ensure optimal dissolution of the bioactive compounds present in the propolis.

After the extraction, the resulting suspensions were carefully filtered sequentially through filter paper (Whatman No. 1, Millipore, USA) to separate the solid residue from the liquid extract of EEP. The filtrates were then concentrated by removing the ethanol solvent under reduced pressure using a rotary evaporator (Rotavapor R-215, Biichi, Switzerland) set at a temperature of 30°C and a vacuum pressure of 995 hPa. The concentrated aqueous residues were subsequently centrifuged at 3900 x g for 10 minutes at 4°C. This step was crucial to separate and remove any insoluble, non-polar components such as waxes that precipitated upon solvent removal, yielding a supernatant. The supernatant, containing the soluble bioactive compounds, was carefully collected. It was then frozen at -80°C and lyophilized using a freeze dryer at -110°C for 48 hours to obtain the final dry EEP powder. The resulting extracts were weighed to determine the extraction yield (Table 3.2), transferred into amber glass vials to protect them from light, and stored at -20°C until further analysis. A schematic of the complete extraction and purification workflow is presented in Figure 3.2.

Table 3.2
The Extraction Yield of Ethanolic Extract of Propolis (EEP) of *G. thoracica.*

No	**Location**	**Initial Mass of Raw Propolis** (g)	**Extraction Yield (g)**
1	SER	10.00	5.56
2	**SA**	10.00	6.38
3	**HB**	10.00	4.23

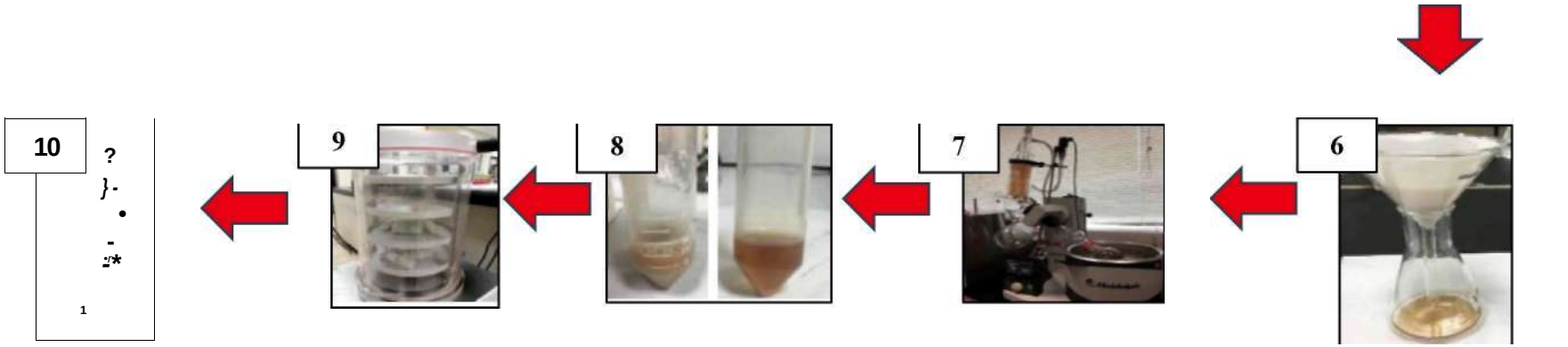

Figure 3.2 Schematic illustration of procedure for preparing the ethanolic extract propolis (EEP).

3.3 Phytochemical Screening of EEP Using Gas Chromatography-Mass Spectrometry (GC-MS)

The chemical screening of the EEP samples were analysed using Gas Chromatography-Mass Spectrometry (GC-MS). The EEP is rich in polar, thermally stable compounds including phenolics, flavonoids, and carboxylic acids, a derivatization step was necessary to enhance their volatility and stability for GC separation and detection. Therefore, each EEP was subjected to a silylation procedure where an active hydrogen such as alcohols, carboxylic or amines, is replaced with a silyl group, typically trimethylsilyl (TMS) or tert-butyldimethylsilyl (t-BDMS). This process transforms polar, less volatile, and thermally unstable compounds into more volatile, less polar, and thermally stable derivatives, making them suitable for GC-MS analysis, following an established protocol (Bankova et al., 2019) with minor modifications.

Approximately, 1 mg of the EEP was combined with 59 uL of pyridine (anhydrous) and 75 uL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as the silylating agent. This reaction converts active hydrogens in hydroxyl (-OH) and carboxyl (-COOH) groups of phenolic and other polar compounds into their corresponding trimethylsilyl (TMS) ethers and esters. The mixture was vortexed and heated at 80°C for 20 minutes in a dry heating block to complete the derivatization. Following the silylation, the silylated sample was filtered through a 0.25 um PTFE syringe filter to remove any particulate matter that could obstruct the analytical process. The filtered sample was then transferred into a vial, preparing it for GC-MS analysis.

The GC-MS analysis was performed at the Instrumentation Laboratory, Level 2, Sarj ana Building, Faculty of Applied Sciences, Universiti Teknologi MARA(UiTM), Shah Alam, Selangor, Malaysia. The analysis was carried out using an Agilent G-59770 system (Agilent Technologies Inc., USA) equipped with an HP-5MS capillary column (30 m x 250 um x 0.25 um film thickness). Helium was utilized as the carrier gas at a constant flow rate of 1.0 mL/min to ensure optimal column performance. The injector temperature was set at 250°C, and a 1 uL of derivatized sample was injected in split mode. The oven temperature program programmed as follows the initial temperature of 80°C held for 1 min, increased to 160°C at 5°C/min, held for 2 min, then ramped to

280°C at 10°C/min and held for 5 min. Compounds were identified by comparing the mass spectral fragmentation patterns and retention times of the eluted peaks with reference spectral data in the National Institute of Standards and Technology (NIST14) mass spectral library.

3.4 Determination of Antioxidant activity

3.4.1 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of propolis extracts was determined using Folin-Ciocalteu method, with gallic acid serving as the standard solution (Sahlan et al., 2018). Briefly, 1 mL of gallic acid was prepared at eight different concentrations (5, 50, 75, 100, 250, 500, 750, and lOOOug/mL). Then, 25 uLof ethanol extract of propolis and the standard solutions were mixed with 100 uL of 25% Folin reagent in a 96-well plate and kept under continuous agitation for four minutes at room temperature. Subsequently, 75 uL of 7.5% sodium carbonate was added to the reaction mixture, shaken for 60 s, and left to stand at room temperature for two hours. The absorbance was measured at 765 nm using a microplate reader. The results of the regression line obtained from the standard curve were used to calculate the TPC value and were expressed as mg/mL Gallic Acid Equivalent (GAE). The test was performed in triplicate.

3.4.2 Determination of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of propolis extracts was determined using the aluminium chloride colorimetric method, with quercetin used as the standard solution (Farasat et al., 2014). Briefly, 1 mL of quercetin at eight different concentrations (5, 50, 75, 100, 250, 500, 750, and lOOOug/mL) were prepared to obtain the standard curve. Then, 20 uL of prepared standard solution and 1 mg/mL of propolis extracts will be added into a 96-well plate, mixed with 20 uL of 10% aluminium chloride solution, 20 uL of 1M potassium acetate, and 140 uL distilled water. The plate was shaken continuously for 1 minute before incubation in the dark for 30 minutes at room temperature. The absorbance was measured at 415 nm using a microplate reader. The TFC value was calculated using a linear regression line plotted on the standard curve

and expressed as mg/mL Quercetin Equivalent (QE). The test was performed in triplicate.

3.4.3 **Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging** Assay

The DPPH free radical scavenging activity of the EEP was evaluated according to the method described by Nafi et al. (2019), with minor modifications. Antioxidant activity was expressed as the half-maximal inhibitory concentration (ICso), defined as the concentration of extract required to scavenge 50% of DPPH free radicals. A 1 mM DPPH stock solution was prepared by dissolving 5 mg of DPPH in 100 mL of methanol. A working solution (200 uM) was obtained by diluting the stock solution with methanol prior to analysis. Quercetin was used as a positive control and reference antioxidant. Stock solutions of EEP samples and quercetin were prepared at a concentration of 1 mg/mL in dimethyl sulfoxide (DMSO).

A series of two-fold serial dilutions were then prepared from these stocks to obtain a final concentration range of 7.8125 to 500 ug/mL (7.8125, 15.625, 31.25, 62.5, 125, 250, and 500 ug/mL). This broad concentration range was selected based on preliminary tests to ensure that the ICso value for each sample would fall within the tested range, allowing for accurate calculation. In a 96-well microplate, 25 uL of each sample or standard concentration was mixed with 200 uL of the 200 uM DPPH working solution. A negative control (blank) was prepared by mixing 25 uL of DMSO with 200 uL of the DPPH solution. The plate was gently agitated and then left to stand in the dark at room temperature for 30 minutes. After incubation, the absorbance of each well was measured at 517 nm using a microplate reader (Thermo Scientific, USA). All experiments were performed in triplicate. DPPH radical scavenging activity was calculated using the following equation:

$$Inhibition\ (\%) = \frac{(Abs\ of\ blank - Abs\ of\ sample)}{Abs\ of\ blank} \times 100\%$$

3.4.4 **Ferric Reducing Antioxidant Power (FRAP)** Assay

The ferric reducing ability of the EEP was determined following the method described by Zarate et al. (2018) with slight modifications. The FRAP reagent was

freshly prepared by combining three solutions. Solution A consisted of 300 mM acetate buffer (pH 3.6), prepared by dissolving 3.12 g of sodium acetate anhydrous in distilled water and adjusting the pH with 1.6 mL of glacial acetic acid to a final volume of 1 L. Solution B was prepared by dissolving 0.031 g of 2,4,6-tripyridyl-s-triazine (TPTZ) in 10 mL of 40 mM hydrochloric acid. Solution C consisted of 20 mM ferric chloride hexahydrate (FeCh 6FL>0), prepared by dissolving 0.054 g of FeCh 6FL>0 in 10 mL of distilled water. The working FRAP reagent was prepared by mixing Solutions A, B, and C in a ratio of 10:1:1 (v/v/v) and pre-warmed at 37 °C for 10 min prior to use. Subsequently, 30 uL of EEP sample was added to the FRAP reagent in a microplate well and mixed thoroughly. The reaction mixture was maintained at 20 °C for 30 min in the dark. Absorbance was measured at 593 nm using a microplate reader. All assays were performed in triplicate. The ferric reducing antioxidant capacity was quantified using a ferrous sulphate calibration curve, and results were expressed as Fe^{2+} equivalents.

3.5 *In Vitro* Assays

3.5.1 Cell Culture and Differentiation

Mouse 3T3-L1 preadipocytes obtained from the American Type Culture Collection (ATCC, CL-173, Manassas, VA, USA) were cultured, maintained, and differentiated as described by Lee et al. (2011). In brief, the cells were plated in a 5% CO2 at 37°C and were grown in high glucose Dulbecco's modified Eagle medium (DMEM) containing 1% penicillin-streptomycin (P/S) and 10% fetal bovine serum (FBS). After reaching 90% confluence, cell differentiation was induced with a different induction medium hormonal cocktail mixture consisting of 0.5 mM 3-isobutyl-l-methylxanthine (TBMX), 1.0 uM dexamethasone (DEX), and 10 ug/mL insulin (designated "day 0"). On day 2, cells were then maintained in regular medium containing 10 ug/mL insulin and were subsequently replaced every 2 days. On day 8, following the induction of differentiation into adipocytes, the cells were treated with the presence of EEP at different concentrations. Cells were harvested for further experiments.

3.5.2 Cell Viability

The MTT assay was used for detection of cell viability and cell cytotoxicity. In brief, the 3T3-L1 preadipocytes were seeded at a density of 1 x 104 cells per well in 96-well plates, and cells were treated with EEP at different concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and lOOOug/mL). Then, the cells were incubated with EEP in a culture medium as described by Choi et al. (2017). The cells were incubated in the dark with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution for 4 hours at 37 °C. The supernatants were aspirated, dimethyl sulfoxide (DMSO) was added to each well, and the plates were agitated to dissolve the formazan crystal product. In this study, N-Acetylcysteine (NAC) was used as a positive control. Absorbance was measured at 570 nm using a multi-well plate reader, and the percentage of viable cells was calculated using the following equation:

$$\textit{Cell viability}\ (\%) = \frac{\textit{(Mean absorbance of each treatment qroup)}}{\textit{(Mean absorbance of control group)}} \times 100\%$$

3.5.3 Lipid Accumulation Assay

The amount of lipid accumulation was measured by ORO staining as described by Choi et al. (2017). In brief, the cultured adipocytes were washed with PBS three times prior to fixation in 10% formaldehyde for 1 hour. Then, they were washed again with PBS three times and allowed to completely dry. The cells were stained with 0.5% Oil Red O solution dissolved in isopropanol (60% v/v) for 30 mins at room temperature, washed four times with water, and dried. Stained cells were monitored under a microscope and quantified via elution with isopropanol. In this study, N-acetylcysteine (NAC) was used as a positive control. The optical density (OD) at 490 nm was then measured. Oil Red O staining was calculated and expressed as a percentage using the following formula:

$$\textit{Lipid accumulation}\ (\%) = \frac{\textit{(Mean absorbance of each treatment qroup)}}{\textit{(Mean absorbance of control group)}} \times 100\%$$

3.5.4 **Nitroblue Tetrazolium (NBT)** Assay

The effect of propolis extract on ROS production was determined by the NBT assay. In brief, 3T3-L1 preadipocytes were grown to confluence and induced to differentiate into adipocytes as described by Choi et al. (2017). ROS production was detected by an NBT assay. NBT is reduced by ROS to a dark blue, insoluble form of NBT called formazan. At 8 days of induction, the cells were incubated in PBS containing 0.2% NBT for 90 min. The formazan was dissolved in 50% acetic acid, and the absorbance was determined at 570 nm. In this study, N-acetylcysteine (NAC) was used as a positive control. The NBT (0.2%) staining solution was prepared in PBS with 20 mg of NBT powder added to PBS (10 ml) and vortexed briefly to dissolve (Javvaji et al., 2020). The NBT assay was calculated and expressed as a percentage using the following formula:

$$ROS(\%) = \frac{(Mean\ absorbance\ of\ each\ treatment\ qroup)}{(Mean\ absorbance\ of\ control\ group} X100\%$$

3.5.5 **Quantitative Real-time PCR (qRT-PCR) Analysis**

Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent after the treatment with 1000, 500, 100, 50, and 10 *uglmL* of propolis extracts. The modified method was described by Choi et al. (2006). First-strand cDNA synthesis was performed with 2 g of total RNA using Superscript II reverse transcriptase. In a fluorescent temperature cycler, ten percent of each reverse transcription (RT) reaction was amplified in a polymerase chain reaction (PCR) containing 4 mM magnesium chloride (MgCb), 4pM each primer, and 1 x LightCycler DNA Master SYBR Green 1 mix. Samples were incubated in the LightCycler for an initial denaturation at 94 °C for 30 s, followed by 40 PCR cycles. Each cycle consisted of 95°C for 10 s, 60 °C for 5 s, and 72 °C for 12 s. The oligonucleotide primers for the experiment are indicated in (Table 3.5.5). To confirm the amplification of specific transcripts, melting curve profiles (cooling the sample to 65 °C for 15 s and heating slowly to 95 °C with continuous measurement of fluorescence) were produced at the end of each PCR. P-actin was used as housekeeping gene, and all experiments were performed three times.

Table 3.5.5

Primer sets for real-time quantitative polymerase chain reaction (RT-qPCR).

Gene	Forward (5'-3')	Reverse (5'-3')
PPARy	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT
C/EBPa	TTACAACAGGCCAGGTTTCC	GGCTGGCGACATACAGTACA
SREBP-1	TGTTGGCATCCTGCTATCTG	AGGGAAAGCTTTGGGGTCTA

3.5.6 Statistical Analysis

The results were expressed as mean ± standard deviation (SD). The data were analysed using one-way Analysis of Variance (ANOVA), followed by Tukey test and Pearson's correlation coefficient test using GraphPad Prism version 7.0 Software (CA, USA). Differences between the experimental groups were considered significant if $P < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Phytochemical Identification using GC-MS Screening

The gas chromatography-mass spectrometry (GC-MS) analysis of ethanolic extract of propolis (EEP) from *G thoracica* collected from three locations in Selangor including Serdang (SER), Shah Alam (SA), and Hulu Bernam (HB) were successfully identified a diverse range of bioactive compounds. A total of 42 distinct compounds were identified across all samples into major classes such as phenolic compounds, terpenoids, sugar alcohols, steroids, fatty acids, and aromatic compounds. The full profile of these constituents, categorized by location, is presented in Table 4.1.

Table 4.1

Comparative phytochemical composition of EEP from three localities (SER, SA, HB) via GC-MS analysis.

SER			SA			HB		
Retention Time (min)	**Compound Names**	**Area (%)**	**Retention Time (min)**	**Compound Names**	**Area (%)**	**Retention Time (min)**	**Compound Names**	**Area (%)**
				Phenolic Compounds				
16.2191	2,6-Bis(tert-butyl)phenol, TMS derivative	0.20	10.7627	2-Methoxy-4-vinylphenol	0.31	23.1196	Phenol, 2-(3,7-dimethylocta-2,6-dienyl)-	4.36
	-		8.2062	Catechol, TMS derivative	0.11	8.2846	Catechol, TMS derivative	0.36
	-		24.5433	2,4-Dihydroxybenzoic acid, 3TMS derivative	0.58	15.3799	2,4-Di-tert-butylphenol	0.37
			23.4618	2,6-Dihydroxyacetophenone, 2TMS derivative	1.46	27.2984	4-Hexylphenol, TMS derivative	0.63
	-		28.2602	5-Methylsalicylic acid, 2TMS derivative	1.46	29.552	Phenol, 3-pentadecyl-	0.79
	-		30.8877	CardanolC17:l(TMS)	0.87		-	
				Terpenoid				
26.3279	Heneicosane	0.25	13.2863	Caryophyllene	0.18	13.3097	Caryophyllene	5.70

			14.0843	1,4,7,-Cy cl oundecatri ene, 1,5,9,9-tetramethyl-, Z,Z,Z-	0.18	32.2124	Squalene	1.55
	-		17.0865	(-)-Globulol	0.11		-	
	-		28.142	Taraxasterol	0.21		-	
				Steroid				
26.2928	5.alpha.-Pregn-16-en-20-one, 3.beta., 12.alpha. dihydroxy-,diacetate	0.21				26.0788	3 .beta.-Hydroxy-5-androsten-17-carboxylic acid	4.39
				Sugar Alcohols				
15.9565	Erythritol, 4TMS derivative	26.6	22.4733	Myo-Inositol, 6TMS derivative	0.94	22.4665	Scyllo-Inositol, 6TMS derivative	0.84
20.6396	Xylitol, 5TMS derivative	0.46	24.727	Scyllo-Inositol, 6TMS derivative	1.36		-	
20.7007	Adonitol, 5TMS derivative	0.19						
26.681	Arabinitol, 5TMS derivative	0.25		-			-	
22.4665	Scyllo-Inositol, 6TMS derivative	1.91		-			-	
26.2144	Myo-Inositol, 6TMS derivative	2.49		-			-	

Sugars								
18.4903	Arabinose, 4TMS derivative	0.26		-			-	
18.8526	L-(+)-Rhamnopyranose, 4TMS derivative	0.72		-			-	
20.4732	D-Lactose, (isomer 1), 8TMS derivative	0.32		-			-	
22.791	D-Fructose, 5TMS derivativ	3.99		-			-	
25.052	D-Glucose, 5TMS derivative	2.97		-			-	
Aromatic Compounds								
25.4617	Benzene, hexakis(dimethylsilyl)-	0.17	24.3949	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	0.22	25.4617	Benzene, hexakis(dimethylsilyl)-	4.36
	-		34.121	6H-Benzo[b]naphtho[2,3-h] carbazole	0.39		-	
	-		6.2831	5-Methyl-2-phenylindolizine	0.18		-	
Fatty Acids								
	-			-		19.3555	Dodecanoic acid, TMS derivative	0.68
	-			-		26.8911	Octadecanoic acid, ethyl ester	1.91

* TMS derivative: trimethylsilyl derivative

The chemical profiles exhibited notable similarities and distinct variations among the three locations, which can be attributed to the diverse botanical sources available to the bees in each location. All three EEP samples shared the presence of phenolic compounds, sugar alcohols and aromatic compounds, highlighting a common phytochemical compound found in *G. thoracica* propolis. This aligns with previous studies identifying these classes as typical constituents of Malaysian stingless bee propolis (Nazir et al., 2018). However, there is the differences observed between location. The extracts from SER were characterized by a high abundance and diversity of sugar alcohols and simple sugars, with Erythritol constituting a peak (26.6%). Sugar alcohols like erythritol and xylitol are related with prebiotic potential and low glycemic impact (Asasta et al., 2024).

It also contained steroids and aliphatic terpenoids not found in the other samples. The phenolic profile dominated by alkylated phenols like 2,6-Bis(tert-butyl)phenol. Phenolic compounds, with their hydroxyl groups, are potent antioxidants that scavenge free radicals and may modulate metabolic pathways (Kumar & Goel, 2019). The extracts from SA profile were diverse in phenolic acids and derivatives, including 2,6-dihydroxyacetophenone, 5-methylsalicylic acid, and cardanol. Significantly, it was the only location where the terpenoid taraxasterol was detected. Taraxasterol is a pentacyclic triterpenoid, characterized by a lupane skeleton, and its identification in this study is based on its characteristic pattern and comparison with library spectra, consistent with its related to anti-inflammatory properties (Zhang et al., 2024).

The chemical structure of taraxasterol is illustrated in Figure 4.2, which features a pentacyclic triterpenoid skeleton known for its significant biological activity. Triterpenoids such as taraxasterol are well-documented for their potent anti-inflammatory and anti-adipogenic properties (Jiao et al., 2022). The absence of taraxasterol in SER and HB is not indicative of lower quality but rather a distinct botanical signature, emphasising the concept of variation based on geography. These findings are constant with literature stating that terpenoids and phenolics are primary bioactive components in stingless bee propolis, responsible for their biological activity (Zhang et al., 2020; Mohd Salim et al., 2018). The synergistic interaction among these diverse compounds likely underpins the overall biological potency of the propolis extracts.

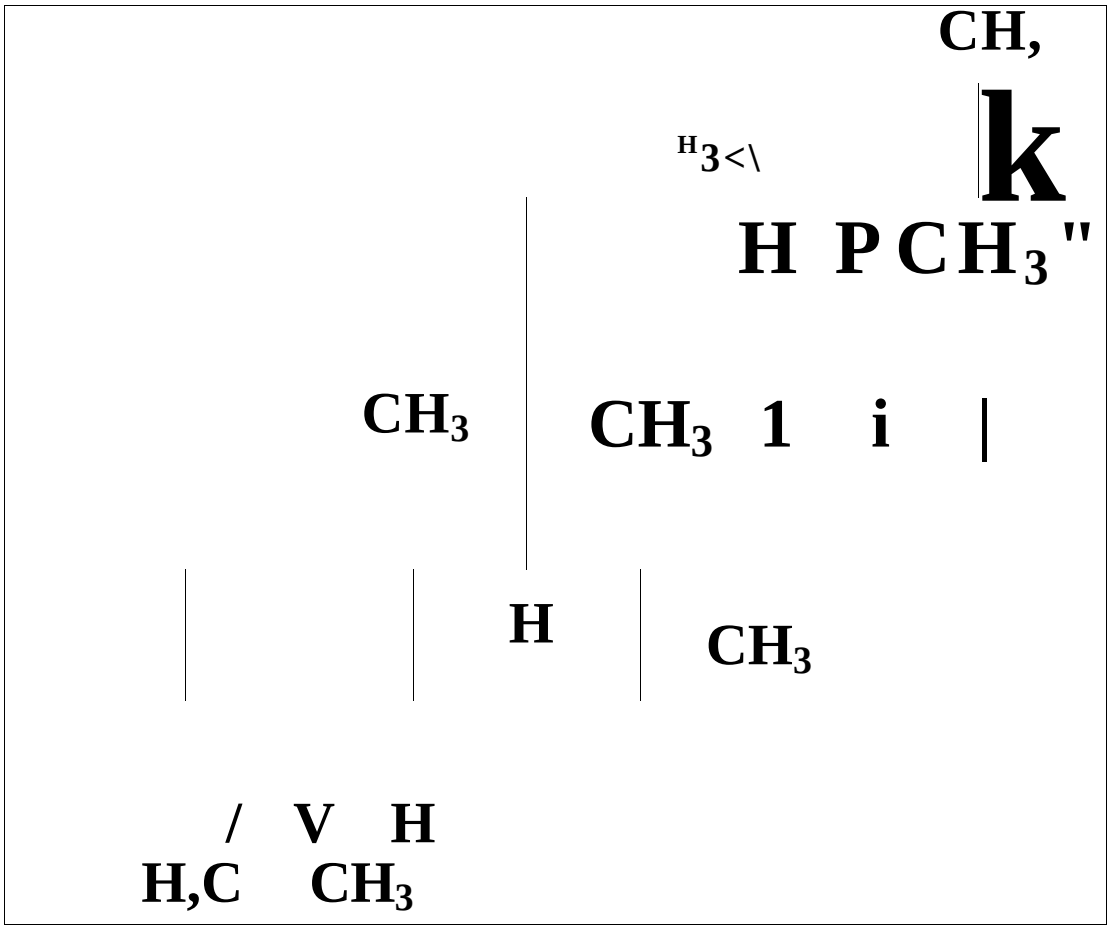

Figure 4.2 The structure of taraxasterol representative bioactive compounds identified in *G thoracica* (Jiao et al., 2022).

The detection of diverse phytochemical constituents in *G thoracica* propolis extracts was facilitated by the derivatization step performed prior to GC-MS analysis. Many phenolic compounds and sugar alcohols are inherently polar and thermally labile, rendering them unsuitable for direct gas chromatographic separation (Moldoveanu & David, 2018). Derivatization using N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) replaces active hydrogen atoms in functional groups such as -OH and -COOH with trimethylsilyl (TMS) groups, thereby enhancing volatility and thermal stability. For example, catechol (CeHeCh) can be converted into its bis-TMS derivative (Ci_2H220_2Si2), allowing its tentative detection by GC-MS (Shareef et al., 2006). This derivatization step enabled the qualitative profiling of a broader range of derivatisable constituents reported in Table 4.1. Without derivatization, such compounds would remain undetected, leading to under-representation of polar phenolics in GC-MS profiles. Variations in compound profiles among samples are likely associated with differences in local floral sources at the respective foraging sites.

Despite these advantages, GC-MS primarily provides qualitative information on volatile and semi-volatile compounds and may not adequately represent non-volatile, high-molecular-weight polyphenols. In this regard, High-Performance Liquid Chromatography (HPLC) is commonly employed for the targeted quantification of specific phenolic compounds, particularly when reference standards are available. However, the primary objective of the present study was exploratory phytochemical screening rather than targeted quantification of predefined compounds. GC-MS was therefore considered appropriate for obtaining a broad qualitative profile of derivatisable constituents in *G thoracica* propolis. The incorporation of HPLC analysis in future studies is recommended to complement GC-MS findings and to enable quantitative characterisation of selected non-volatile phenolic markers.

4.2 Total Phenolic Contents (TPC) and Total Flavonoid Contents (TFC)

The TPC and TFC of EEP are presented in Table 4.2. The gallic acid standard curve were used to calculate the TPC values with a regression line of $y = 0.0024x + 0.0477$, ($R^2 = 0.993$). The TPC of EEP ranged between 111.38 - 302.21 mg/mL gallic acid equivalents (GAE). The highest TPC value observed in EEP from SER (302.21 ± 0.11 mg/mL GAE), followed by SA (156.79 ± 0.06 mg/mL GAE), while HB exhibited the lowest TPC value (111.38 ± 0.06 mg/mL GAE). There were significant differences ($p < 0.05$) in the TPC of EEP from *G thoracica* when compared with propolis samples from different locations. A previous study by Kustiawan et al. (2022) reported that TPC produced by stingless bee *G thoracica* from Indonesia exerted the highest TPC with 880 mg GAE/lOOg. A finding by Asem et al. (2019) reported a TPC produced by stingless bee *G thoracica* possesses value with 55.16 ± 7.52 (uM) per g. Other findings were reported by Badiazaman et al. (2019) showed a variation in the TPC value of stingless bee *G thoracica* propolis, ranging from 9.23 - 23.43 mg GAE/g. Additionally, a study conducted by Adli et al. (2022) demonstrated that the propolis produced by stingless bee *G thoracica* possesses the TPC value with 259.84 ± 4.97 mg/mL GAE. The differences in phenolic content values within propolis sample may be attributed to the origin of the propolis raw material (Machado et al., 2016).

The TFC values of EEP was obtained from the quercetin standard curve to calculate the TFC values with the regression line equation of $y = 0.0025x + 0.0767$, ($R^2 = 0.994$). The TFC values of the EEP ranged from 64.68 - 99.08 mg/mL QE. The results indicated that the highest TFC value was observed in the EEP from SER (99.08 ± 0.03 mg/mL QE), followed closely by HB (73.08 ± 0.01 mg/mL QE). In contrast, SA exhibited the lowest TPC value (64.68 ± 0.02 mg/mL QE). Notably, there was a significant difference ($p < 0.05$) of EEP from SER, between SA and HB. In recent study by Adli et al., (2022) it was reported that *G thoracica* propolis exerted the highest TFC value at 435.00 ± 6.57 mg/mL QE. These was accordance with the findings by Mohamed et al. (2016), who reported that Malaysian propolis extracts possesses value with 209.83 ± 1.42 mg quercetin Eq per g. Another study conducted by Badiazaman et al. (2019) highlighted the variability of TFC content of *G thoracica* propolis, ranging from 9.52 to 17.22 mg QE/g.

Table 4.2

The total phenolic and flavonoid contents of EEP from three localities (SER, SA, HB).

Location	Total Phenolic Content (mg/mL GAE)	Total Flavonoids Content (mg/mL QE)
SER	302.21 ± 0.11^{c}	99.08 ± 0.03^{b}
SA	156.79 ± 0.06^{b}	64.68 ± 0.02^{a}
HB	111.38 ± 0.06^{a}	73.08 ± 0.01^{a}

Results are expressed as mean ± SD (n=3). Values with different superscript letters (a-c) in a column differ significantly ($p<0.05$). GAE: Gallic acid equivalent; QE: Quercetin equivalent.

The high TPC in SER (302.2 mg GAE/mL) appears contradictory to its GC-MS profile, which showed only one major phenolic compound (2,6-Bis(tert-butyl)phenol, 0.20%). This inconsistency can be attributed to differences between methodological techniques. The silylation process in GC-MS is optimal for volatile and semi-volatile compounds, and phenolic compounds may not derivatize with equal efficacy (Ncongwane et al., 2023). Many complex or polar polyphenols and flavonoids may not derivatize efficiently, leading to their non-detection. In contrast, their presence could be captured by the colorimetric TPC assay, which reacts with any reducing hydroxyl group on phenolic rings, capturing both complex and simple phenolic compounds. Therefore, the TPC results suggest the presence of a wide array of non-volatile phenolic compounds in SER not fully revealed by GC-MS. This is likely due to a potentially diverse pool of phenolic compounds and the effect of a synergistic substance, where abundant sugar alcohols influence the activity of phenolics (Duque-Soto et al., 2023).

4.3 DPPH Free Radical Scavenging Activity

Table 4.3 demonstrate the percentage inhibition and the ICso values of EEP at concentrations ranging from 7.813 ug/mLto 500 ug/mL, using quercetin as a reference standard. In general, all tested extracts exhibited dose-dependent free radical scavenging activity on DPPH assay. Among the tested samples, propolis from HB exhibited the highest DPPH inhibition (84.82% ± 18.7), followed by propolis from SER (82.51% ± 1.27) and propolis from SA (81.38% ± 30.1). Interestingly, the propolis sample from SER displayed the lowest ICso (25.27 ug/mL), followed by SA and HB, at 65.37 ug/mL and 122.7 ug/mL, respectively. The lower ICso value is indicative of stronger antioxidant activities, as emphasized in studies by Kurek-Gorecka et al. (2022). Meanwhile, the standard quercetin showed the best DPPH inhibitory effect at 90.08% ± 9.76 with ICso value of 18.93 ug/mL. The results of the study highlight the variances in antioxidant activity among the *G thoracica* propolis samples.

As a previous study on propolis *G thoracica* exhibited a range of ICso values between 104.20 - 1009.00 ug/mL (Adli et al., 2022). Another propolis study conducted at different locations in Malaysia revealed ICso value for DPPH test on *G thoracica* within the range between 53 to 190 ug/mL (Badiazaman et al., 2019). In contrast, Ibrahim et al. (2016) reported the free radical scavenging activity of propolis produced

by stingless bees *H. itama* and *G thoracica*, revealing that propolis from *H. itama* exhibited higher antioxidant activity with an ICso value of 15.0 ug/mL compared to propolis from *G thoracica* with an ICso value of 270.0 ug/mL. These variations are likely attributed to the differences in chemical composition in the propolis extracts (Socha et al., 2015). Hence, the utilization of DPPH assay offers several advantages in understanding the antioxidant capacity of the propolis samples. This method proves valuable in quickly assessing antioxidant activity, thereby facilitating the evaluation of the potential of natural compounds.

Table 4.3

DPPH Inhibition percentage and ICso value of EEP from three localities (SER, S A, HB).

	DPPH Inhibition (%)	**ICso DPPH (ng/mL)**
SER	82.51 ± 1.27	25.27 [a]
SA	81.38± 30.1	65.37 [b]
HB	84.82 ± 18.7	122.7 [c]
Quercetin	90.08 ±9.76	18.93 [a]

Results are expressed as mean ± SD (n=3). Values with different superscript letters (a-c) in a column differ significantly ($p<0.05$).

4.4 **Ferric Reducing Antioxidant Power (FRAP)** Assay

The results presented in Table 4.4 demonstrated the FRAP values of EEP at concentrations ranging between 0 to 1000 ug/mL, using gallic acid as standard. The determination of FRAP value of these extracts was determined by constructing a standard curve of iron (II) sulphate heptahydrate (FeSO^FbO). The FRAP values were calculated by comparing the absorbance of ethanolic extract of propolis with the standard curve. The obtained results revealed that the FRAP values of EEP varied from 308.20 ± 0.02 uM Fe^{2}+ to 727.53 ± 0.09 uM Fe^{2}+. Among the test samples, the highest FRAP values were observed was in the EEP from SER (727.53 ± 0.09 uM Fe^{2}+), followed by SA (692.73 ± 0.05 uM Fe^{2}+), while HB exhibited the lowest FRAP value (308.20 ± 0.02 uM Fe^{2}+). Notably, there is a significant difference ($p < 0.05$) of EEP from HB, between SER and SA.

The utilization of gallic acid as a standard showing the highest TPTZ scavenging activity (1708.20 ± 0.16 uM Fe^{2}+). This result was in accordance with a study reported by Asem et al. (2019) propolis produced by stingless bee *G thoracica* exhibited a FRAP value with 587.044 mM Trolox/g. Therefore, the FRAP method was selected in this study since it has been widely used for assessing antioxidants and the reducing power of diverse samples, including propolis (Kurek-Gorecka et al., 2022). The mechanisms of antioxidant action in the FRAP assay involves an electron donor, and the oxidation chain reaction is halted by reducing the oxidized to their stable forms (Bibi Sadeer et al., 2020). The reaction involved the reduction of the Fe^{3}+ TPTZ complex to a blue-coloured Fe^{2}+ TPTZ by an antioxidant agent (Mihai et al., 2011). A higher FRAP value signifies a greater antioxidant capacity.

Table 4.4

FRAP values of EEP from three localities (SER, SA, HB).

	FRAP values (uM Fe^{2}+)
SER	727.53 ± 0.09 [b]
SA	692.73 ± 0.05 [b]
HB	308.20 ± 0.02 [a]
Gallic Acid	1708.20 ± 0.16 [c]

Results are expressed as mean ± SD (n=3). Values with different superscript letters (a-c) in a column differ significantly ($p<0.05$).

The greater the antioxidant activity (lowest DPPH ICso, highest FRAP) of the SER extract correlates with its highest TPC and TFC values. While GC-MS screening showed that phenolic profile appears limited, due to the synergistic interaction of its abundant sugar alcohols with its phenolic constituents may enhance overall reducing power and radical scavenging activity (Loncaric et al., 2018). The SA extract, containing diverse phenolic acids and the triterpenoid Taraxasterol, showed intermediate activity. The HB extract, richer in fatty acids and squalene but lower in phenolics, exhibited the least antioxidant capacity, underscoring the primary role of phenolic compounds in these assays.

4.5 Effect of EEP on Cell Viability in 3T3-L1 Cells

In order to determine the cytotoxicity effects of EEP that influence the cell viability for treating the 3T3-L1 preadipocytes, the MTT assay was performed as presented in Fig. 4.5. Result showed that all eight different concentrations (1000, 500, 250, 125,62.5,31.25, 15.625, and 7.8125 ug/mL) of EEP from three different locations did not have any cytotoxic effects against preadipocytes, as cell viability remained consistently above 80% throughout all tested concentrations, indicating no cytotoxicity. Thus, non-toxic concentrations of EEP were used for all subsequent experiments using 3T3-L1 adipocytes. In light of these results, five non-toxic doses were selected for further experimentation, reflecting a balanced consideration of concentration ranges and cellular population management. Our findings resonate with earlier research conducted by Ikeda et al. (2011), which similarly reported no cytotoxicity in 3T3-L1 cells subjected to Brazilian propolis extracts at concentrations up to 50 ug/mL.

Conversely, Lio et al. (2010) documented a decrease in cell viability at 100 ug/mL when utilizing Brazilian red propolis extract on 3T3-L1 cells. The discrepancies in cytotoxic outcomes between these studies may be attributed to the variations in the chemical compositions inherent to different propolis types, which can significantly vary based on geographical origins, botanical sources, and extraction methodologies employed. Importantly, the absence of cytotoxicity observed in our study suggests that EEP does not adversely affect essential cellular functions, including metabolic activity and mitochondrial integrity, as gauged by the MTT assay, which measures mitochondrial dehydrogenase activity. This finding is critical for evaluating the

suitability of potential therapeutic agents targeting obesity, especially as compounds that disrupt mitochondrial function can lead to unintended adverse effects on cellular energy metabolism. Notably, adipocyte differentiation is inherently an energy-intensive process that requires robust mitochondrial activity. Thus, the demonstrated non-cytotoxic nature of EEP at the concentrations tested reinforces its potential application in the modulation of adipogenesis and its viability as a candidate for obesity management.

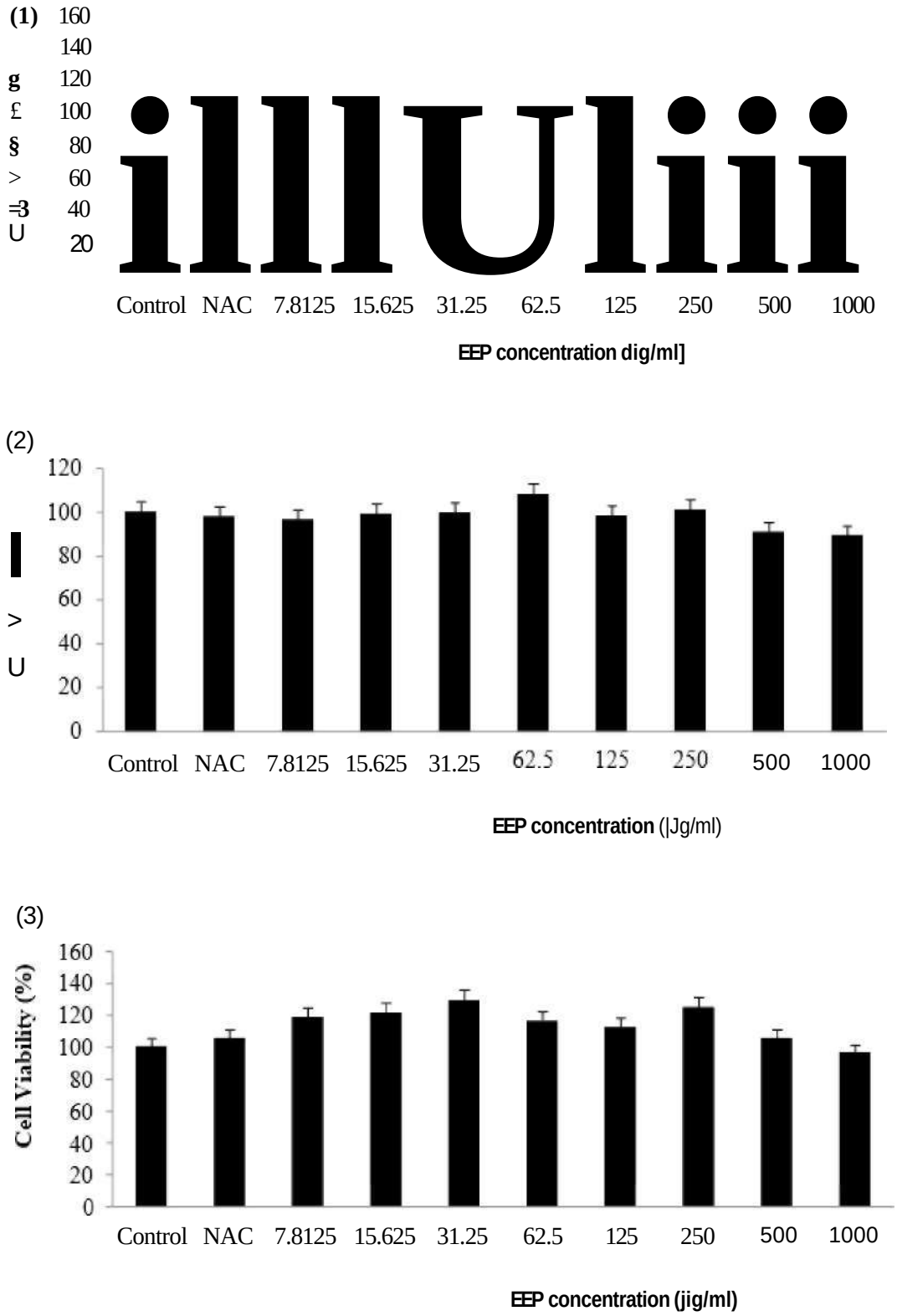

Figure 4.5 Effect of EEP from location (1) SER, (2) SA, and (3) HB on the viability of 3T3-L1 adipocytes. Cells were treated with different concentration of EEP (7.8125 to 1000 ug/mL). Control group represents cells treated with 0.5% DMSO and 5 mM NAC as positive control. Results are represented as mean ± SD of three independent experiments.

4.6 Effect of EEP on Lipid Accumulation in 3T3-L1 Cells

The effect of EEP on intracellular lipid accumulation was comprehensively evaluated using Oil Red O (ORO) staining, a well- established method for visualizing and quantifying lipid droplets within adipocytes. The lipid quantification assay confirmed the decrease of fat accumulation during the adipocyte maturation along treatment with EEP at varied concentrations of 1000, 500, 100, 50 and 10 ug/mL (Figure 4.6A). Microscopic examination of the stained cells revealed a consistent and gradual reduction in the number of lipid droplets as the concentration of EEP was increased (Figure 4.6B). Notably, when evaluating the concentrations from 1000 to 50 ug/mL, EEP demonstrated lipid reduction effects comparable to the positive control, N-acetylcysteine (NAC) ($p>0.05$).

(4.6A)

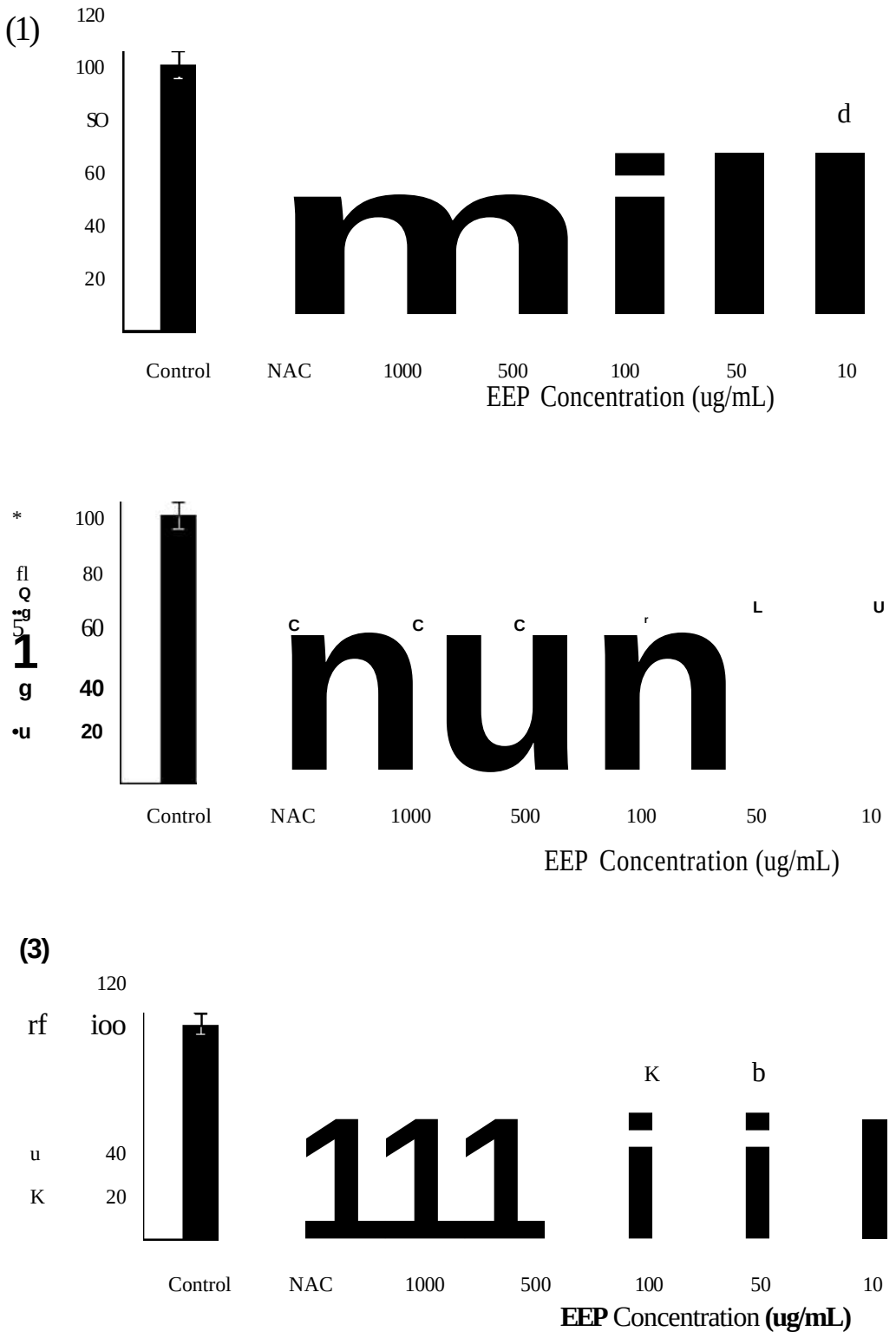

Figure 4.6(A): Quantification of lipid accumulation (%) in 3T3-L1 adipocytes treated with EEP from (1) SER, (2) SA, and (3) HB. Results are represented as mean ± SD from three independent experiments. Bars with different superscript letters (a, b, c, d) indicate statistically significant differences ($p<0.05$).

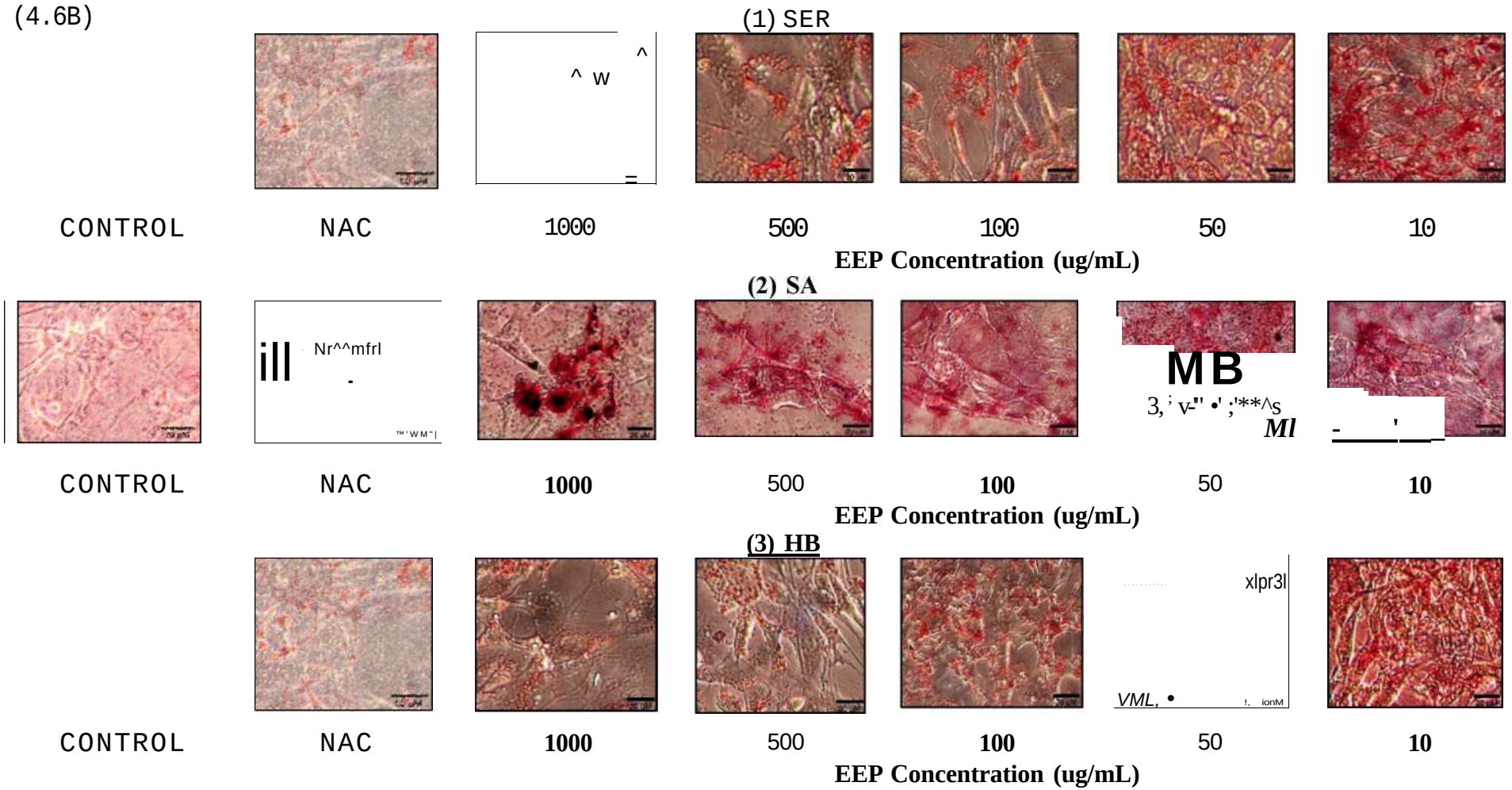

Figure 4.6(B): Microscopic images (400x) of lipid accumulation in 3T3-L1 adipocytes treated with EEP from (1) SER, (2) SA, and (3) HB. The control group represents cells treated with 0.5% DMSO, with 5mM NAC as the positive control. Results are represented as mean ± SD from three independent experiments. Red staining indicates intracellular lipid droplets.

These compelling results indicate that EEP is highly effective in inhibiting adipogenesis in 3T3-L1 cells. The observed dose-dependent nature of this inhibition, particularly pronounced at higher concentrations of EEP, suggests a potential disruption in the bioenergetic and metabolic pathways involved in lipid synthesis and storage processes. Prior research has established that propolis extracts rich in bioactive compounds, such as phenolic acids and flavonoids, possess the capability to modulate adipogenesis by interfering with key signaling pathways that are crucial for fat cell differentiation (Khalilpourfarshbafi et al., 2014). For instance, according to Daleprane and Abdalla (2013), these bioactive constituents can disrupt the differentiation of preadipocytes into mature adipocytes, thereby effectively reducing lipid storage capacity.

Quercetin, a prominent phenolic compound recognized for its presence in propolis, has been documented to inhibit lipid accumulation throughout the adipogenic differentiation process (Khalilpourfarshbafi et al., 2014). The evident dose- dependent reduction in lipid accumulation observed in our study reinforces the premise that EEP plays a significant role in regulating lipid metabolism, with potential at managing obesity. The mechanism contributing to this observed effect is linked to the inhibition of lipid droplet formation through the modulation of gene expression involved in lipid uptake and storage, specifically those genes encoding fatty acid- binding protein 4 (FABP4) and perilipin 1 (PLIN1) (Daleprane and Abdalla, 2013). Additionally, the anti-adipogenic effect of EEP may also be mediated through its ability to influence oxidative stress and inflammatory pathways, as increased oxidative stress levels have been associated with enhanced lipid accumulation and adipogenic responses.

4.7 Effect of EEP on ROS Production in 3T3-L1 Cells

Reactive oxygen species (ROS) are increasingly recognized for their significant role in mediating adipocyte differentiation and stimulating lipid accumulation within preadipocytes. To evaluate the impact of EEP on ROS generation during the process of adipogenesis, we employed the nitroblue tetrazolium (NBT) assay. The results derived from this assay uncovered a substantial and significant reduction in ROS levels in a dose-dependent manner following EEP treatment at both high and lower concentrations (1000, 500, 100, 50 and 10 ug/mL). Notably, at the higher concentrations of EEP (100-

1000 ug/mL), the inhibition of ROS formation was comparable to that observed with NAC treatment ($p>0.05$), suggesting that EEP possesses antioxidant properties that effectively counteract oxidative stress during adipogenesis (Fig 4.7).

These findings align with existing literature that has highlighted the antioxidant potential of propolis extracts. For instance, a study conducted by Zullkiflee et al. (2022) demonstrated that Malaysian propolis exhibited profound free radical- scavenging activity, allowing it to effectively neutralize ROS and reduce oxidative stress in adipocytes. Similarly, Brazilian propolis has been extensively investigated and shown to prevent ROS-induced adipogenic differentiation by inhibiting key redox- sensitive transcription factors, including nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor-kappa B (NF-KB) (Santos et al., 2009). The ability of EEP to suppress ROS formation indicates its potential to interfere with pathways mediated by oxidative stress that promote adipogenesis and lipid accumulation.

Oxidative stress has been implicated in the pathophysiology of obesity and metabolic syndrome due to its role in instigating chronic inflammation and insulin resistance. By effectively reducing ROS levels, EEP may contribute to the maintenance of cellular redox homeostasis, thereby offering protection against obesity-related metabolic disorders. This highlights the promising potential of *G. thoracica* propolis as a functional food ingredient endowed with both anti-obesity and antioxidant properties, further supporting its integration into dietary strategies aimed at enhancing metabolic health and combating obesity.

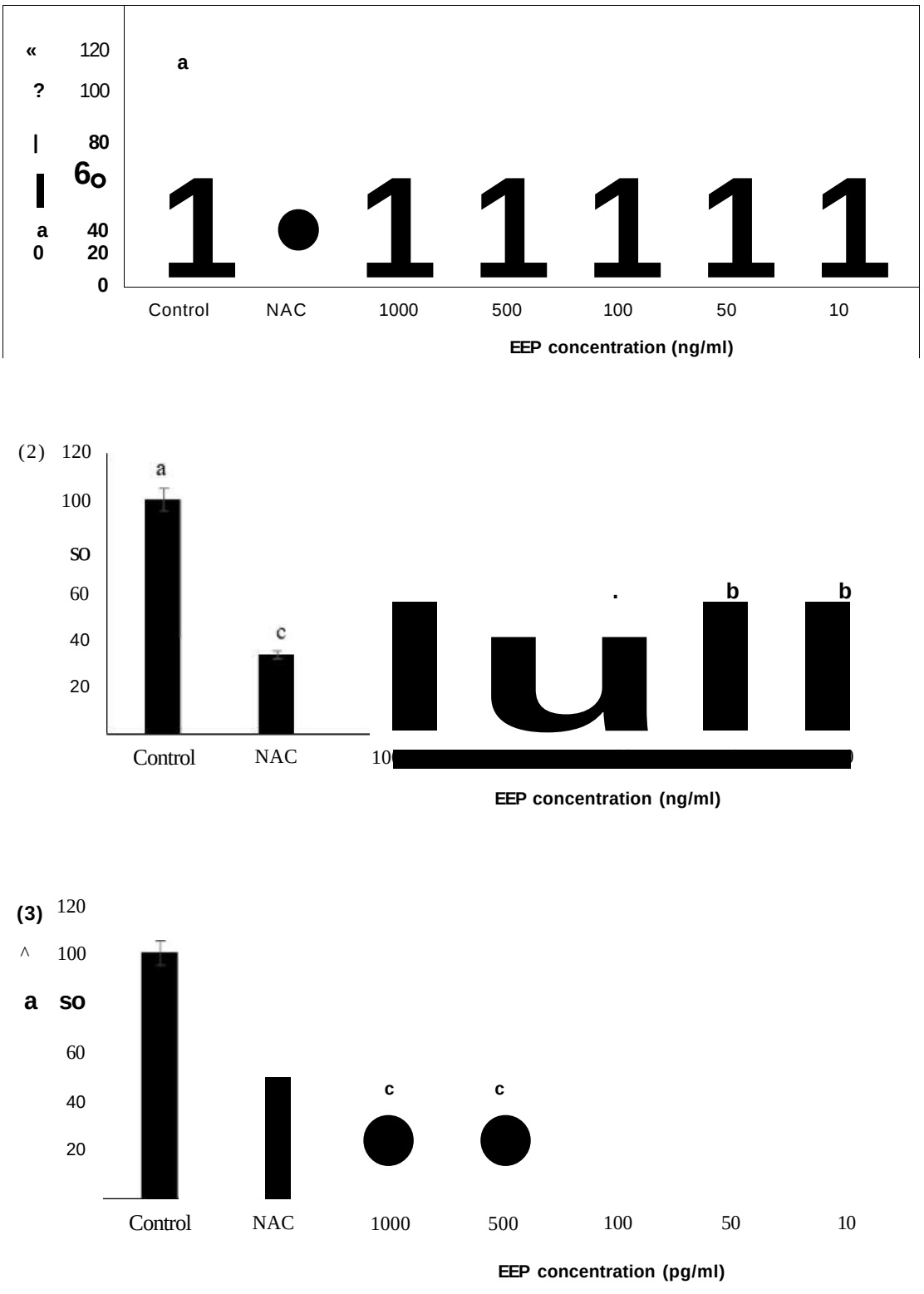

Figure 4.7 Effect of EEP from (1) SER, (2) SA, and (3) HB on intracellular ROS production in differentiating 3T3-L1 cells. Control group represents cells treated with 0.5%DMSO and 5 mMNAC as positive control. Results are represented as mean ± SD of three independent experiments. Bars with different superscript letters (a, b, c) are significantly different ($p<0.05$).

4.8 Effect of EEP on the Expression of Adipogenic Transcription Factors in 3T3-L1 Cells

The process of adipogenesis is intricate and tightly regulated, primarily controlled by pivotal transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR-y), CCAAT/enhancer-binding protein alpha (C/EBP-a), and sterol regulatory element-binding protein-1 (SREBP-1). These transcription factors are integral to promoting adipocyte differentiation and facilitating lipid accumulation by regulating a cascade of downstream target genes responsible for fatty acid synthesis and storage. To elucidate the effect of EEP on adipogenic transcription factor expression, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed.

The results indicated a significant, dose-dependent suppression of PPAR-y, C/EBP-a, and SREBP-1 expression in cells treated with EEP, particularly in comparison to the control group that did not receive any treatment. Notably, at the higher concentrations (500 and 1000 ug/mL), EEP exhibited inhibitory effects that were comparable to those induced by NAC treatment ($p>0.05$), indicating that EEP appears to function as a potent suppressor of adipogenic gene expression (Figure 4.8). These findings are consistent with studies that have explored the effects of propolis on adipogenesis. For example, extracts of Brazilian propolis, which are rich in caffeic acid phenethyl ester (CAPE), have been demonstrated to significantly downregulate the expression of key adipogenic transcription factors, specifically PPAR-y and C/EBP-a, resulting in reduced TG accumulation in 3T3-L1 cells (Kitamura et al., 2013).

Likewise, investigations on Chinese propolis have revealed that flavonoids such as chrysin and galangin inhibit adipogenic differentiation by modulating pathways associated with PPAR-y signaling (Yang et al., 2005). The molecular mechanisms that underlie the anti-adipogenic effects of EEP may involve multiple signaling pathways. One compelling mechanism is the inhibition of the mitogen-activated protein kinase (MAPK) pathway, which plays a significant role in adipocyte differentiation. Studies have documented that polyphenols present in propolis can effectively suppress the activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase

(INK), thereby leading to reduced expression of both PPAR-y and C/EBP-a (Megantara et al., 2025).

Moreover, the AMP-activated protein kinase (AMPK) pathway may also contribute to this mechanism, as investigations have shown that propolis extracts can activate AMPK, resulting in diminished lipid accumulation and enhanced fatty acid oxidation (Reddy, 2023). In light of these compelling findings, it is reasonable to conclude that the bioactive compounds found within *G thoracica* propolis exert their anti-adipogenic effects by targeting multiple signaling pathways that are involved in lipid metabolism and adipogenesis. Further investigations utilizing proteomics and metabolomics approaches would be highly beneficial in elucidating the precise molecular targets and mechanisms of EEP, thus providing a more comprehensive understanding of its potential therapeutic applications in the management of obesity.

Gene Expression (Fold)
PPAR-Y
C/EBPA
SREBP-1

Gene Expression (Fold
0.0
0.2
0.4
0.6
0.8
1.0
1.2
PPAR-Y
C/EBPA
SREBP

Gene Expression (Fold)
WSBMm-tPJL
P[Vj
SREBP-

CHAPTER 5
CONCLUSION AND RECOMMENDATION

This study successfully achieved its objectives by providing a comparative evaluation of the phytochemical profiles, antioxidant activities, and anti-adipogenic effects of EEP derived from *G thoracica* collected from three distinct locations in Selangor, Malaysia, namely Serdang (SER), Shah Alam (SA), and Hulu Bernam (HB). The findings clearly demonstrate that geographical origin plays a significant role in influencing the chemical composition and biological activities of stingless bee propolis.

GC-MS-based phytochemical screening revealed both similarities and differences among the three propolis samples. All locations shared common classes of compounds, including phenolic derivatives, terpenoids, sugar alcohols, and aromatic compounds, indicating a conserved chemical signature of *G thoracica* propolis. However, notable location-specific variations were observed. The SER and SA samples exhibited a greater diversity of phenolic-related compounds, whereas the HB sample was characterised by a higher relative presence of fatty acids and non-phenolic constituents. The occurrence of marker compounds such as taraxasterol in SA and squalene in HB further highlights the influence of surrounding vegetation and foraging environment on propolis composition.

In terms of antioxidant properties, the SER extract consistently demonstrated superior activity, as evidenced by the highest total phenolic content (302.21 mg/mL GAE), total flavonoid content (99.08 mg/mL QE), strongest ferric reducing antioxidant power (727.53 uM Fe^{2+}), and the lowest DPPH radical scavenging ICso value (25.27 ug/mL). Although GC-MS screening detected a limited number of derivatisable phenolic compounds in SER, the elevated TPC and antioxidant capacity indicate the presence of non-volatile or poorly derivatised phenolic constituents that were not fully captured by GC-MS analysis. This finding underscores the complementary nature of colorimetric antioxidant assays and chromatographic screening techniques

Beyond antioxidant activity, EEP, particularly from SER, exhibited pronounced anti-adipogenic effects in the 3T3-L1 adipocyte model. The SER extract significantly reduced lipid accumulation and intracellular reactive oxygen species levels, while also downregulating the expression of key adipogenic transcription factors, including PPAR-y, C/EBP-a, and SREBP-1. These results suggest that the biological activities of *G thoracica* propolis are closely linked to its overall phytochemical composition rather than to individual compounds alone, likely arising from synergistic interactions among multiple bioactive constituents.

In conclusion, this study confirms that *G thoracica* propolis exhibits location-dependent variations in phytochemical composition, antioxidant capacity, and anti-adipogenic activity. Among the samples evaluated, the SER propolis extract demonstrated the most favourable biological profile. These findings support the importance of considering geographical origin in the quality assessment and standardisation of stingless bee propolis for functional food or nutraceutical applications. Future research is recommended to incorporate targeted chromatographic techniques, such as HPLC, to quantitatively characterise non-volatile phenolic constituents and to further elucidate the molecular mechanisms underlying the observed bioactivities.

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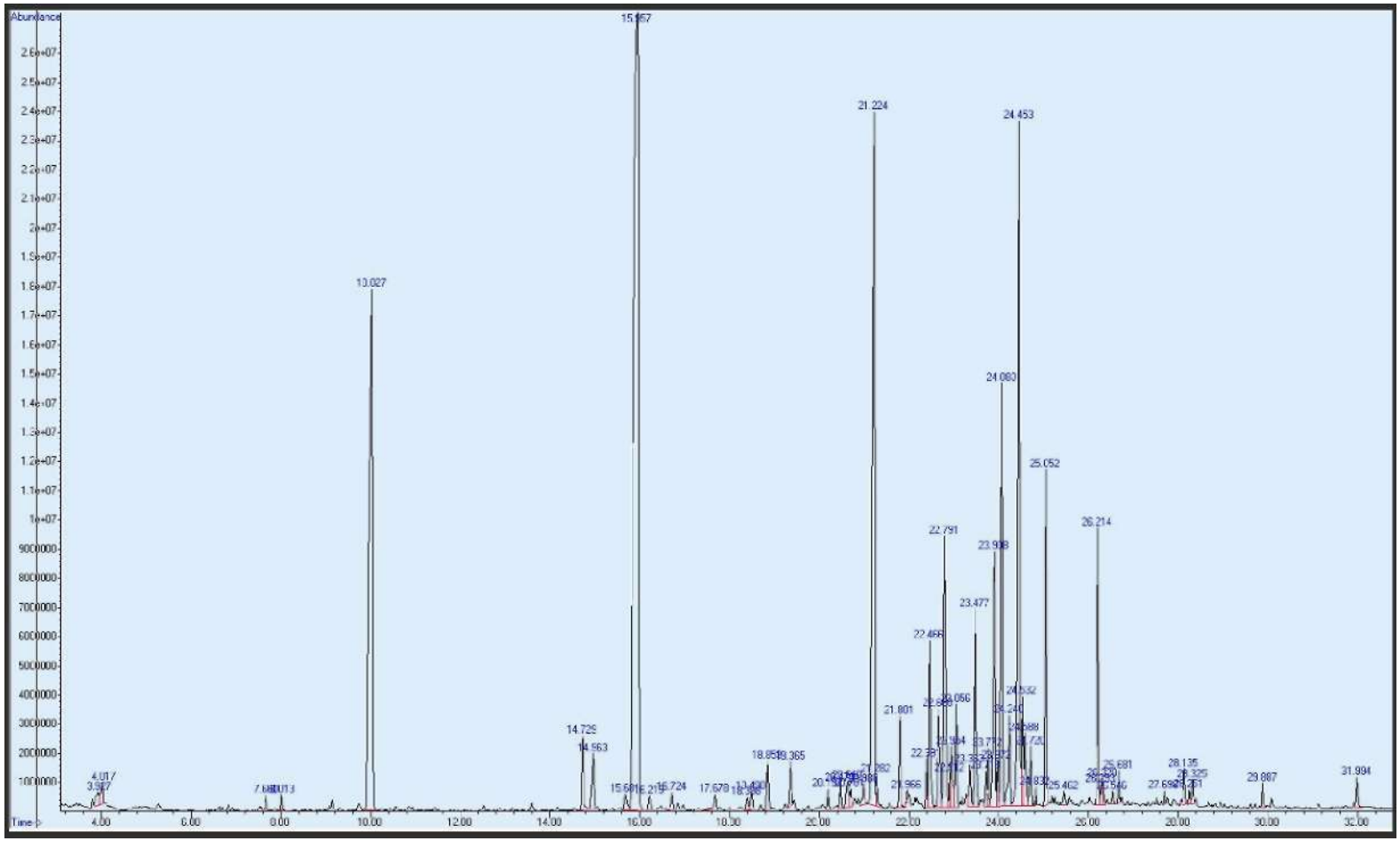
APPENDICES

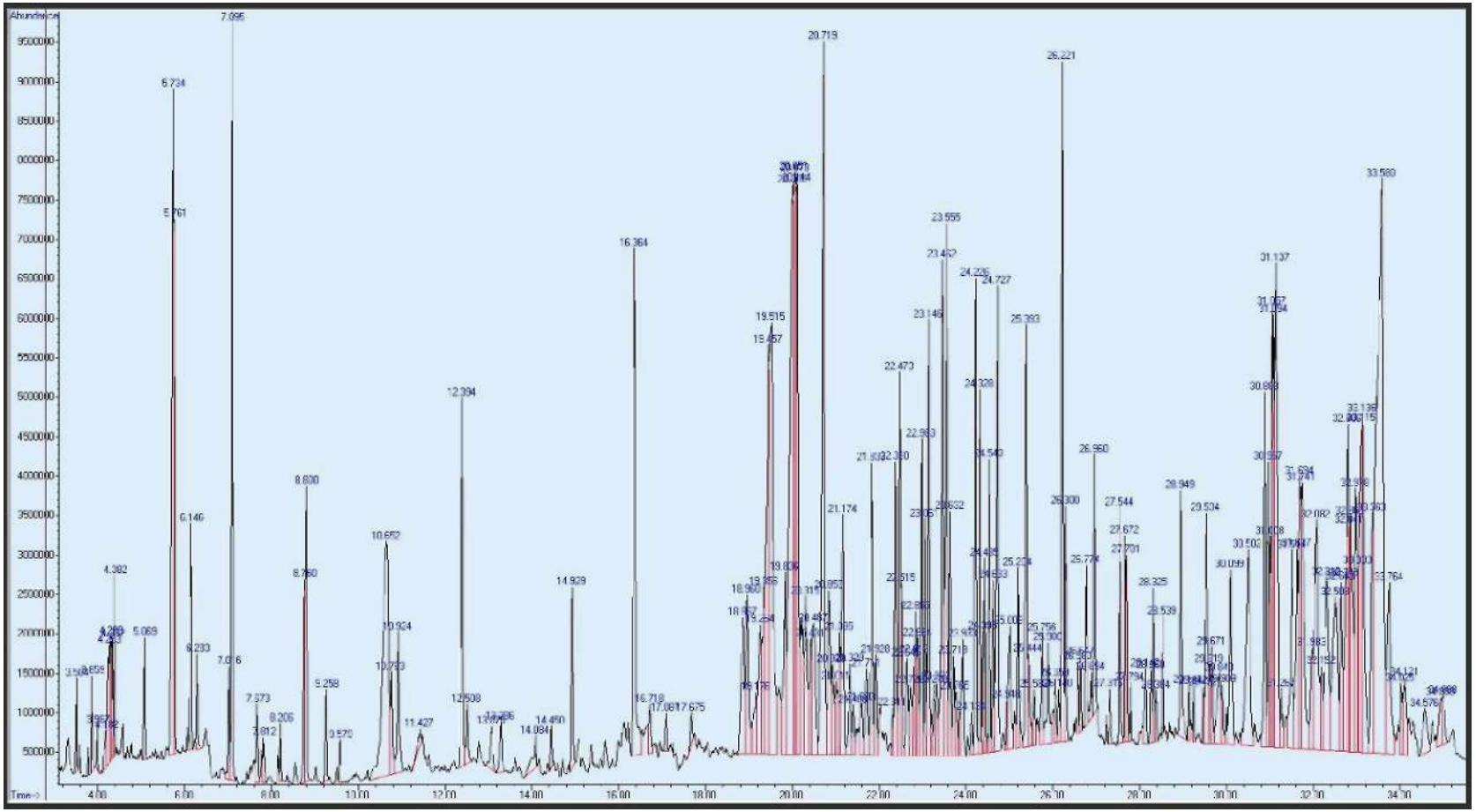
APPENDIX 1

The GC-MS chromatogram of SER



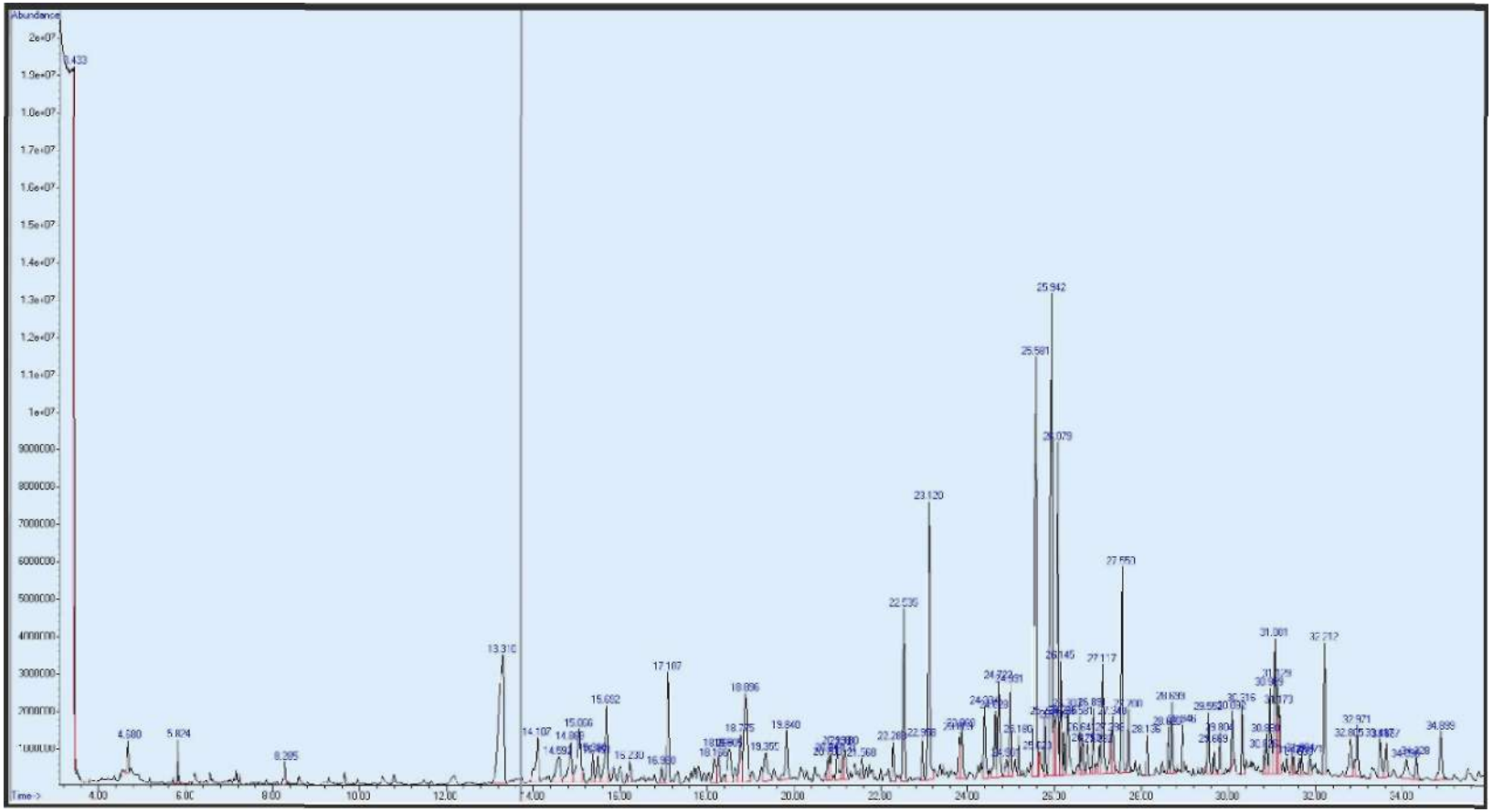
APPENDIX 2

The GC-MS chromatogram of SA



APPENDIX 3

The GC-MS chromatogram of of HB



AUTHOR'S PROFILE

Lini binti Idris graduated with a Bachelor of Applied Science (Hons.) in 2021 from Universiti Malaysia Terengganu. During her undergraduate studies, she completed her final year project titled A Review on the Dietary and Chemical Composition of Seaweed Caulerpa (C. *Racemosa and C. Lentillifera),* which gained her interest in exploring the phytochemical composition and antioxidant potential of propolis produced by *G thoracica.* Currently, she is engaged in a research project focusing on the GC-MS Phytochemical Screening, antioxidant, and anti-Adipogenic Activities of *Geniotrigona thoracica* Propolis Extract from Different Locations in Malaysia.

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Idris, L., Adli, M. A., Mokhtar, S. M., Jamil, N. M., Eshak, Z., Ab Ghani, N., & Mohd Zohdi, R. (2025). Chemical profiling and anti-adipogenic effects of *Geniotrigona thoracica* propolis extract in 3T3-L1 cells. *Malaysian Journal of Chemistry,* 27(1), 134-144.

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