IN VITRO EVALUATION OF THE ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TUALANG HONEY SAMPLES AND PURIFIED 5-HYDROXYMETHYLFURFURAL

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***IN VITRO* EVALUATION OF THE ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TUALANG HONEY SAMPLES AND PURIFIED 5-HYDROXYMETHYLFURFURAL**

by

DANESH A/L CHANDIRAN

Dissertation submitted in partial fulfilment of the requirements for the degree of Bachelor of Biomedical Science (Honours)

January 2025

CERTIFICATE

This is to certify that the dissertation entitled "*In vitro* evaluation of the antioxidant and antimicrobial activities of tualang honey samples and purified 5-hydroxymethylfurfural" is the bona fide record of research work done by Mr. Danesh A/L Chandiran during the period from August 2024 to January 2025 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research, and promotional purposes.

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LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
g	Gram
g/kg	Gram per kilogram
mg/kg	Milligram per kilogram
μg	Microgram
mg	Milligram
mL	Millilitre
mg/L	Milligrams per litre
mg/mL	Milligrams per millilitre
mm	Millimetre
mS/cm	Millisiemens per centimeter

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATH-24	Aged tualang honey of 24 months
ATH-48	Aged tualang honey of 48 months
CUPRAC	Cupric reducing antioxidant capacity
DMSO	Dimethyl Sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPH-H	Hydrazine
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FAMA	Federal Agricultural Marketing Authority
FC	Folin–Ciocalteu
FRAP	Ferric reduction of antioxidant power
FTH	Fresh tualang honey
HAT	Hydrogen Atom Transfer
HbS	Hemoglobin Sickle
HMF	5-Hydroxymethylfurfural
IC_{50}	Half Maximal Inhibitory Concentration
MDR	Multidrug Resistant
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MOH	Ministry of Health
ORAC	Oxygen Radical Absorbance Capacity
PPSK	Pusat Pengajian Sains Kesihatan
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SET	Single Electron Transfer
SMF	5-sulfoxy-methylfurfural
TRAP	Total peroxyl radical trapping antioxidant parameter
TOSC	Total oxyradical scavenging capacity
UPMS	Unit Pengurusan Makmal Sains
USM	Universiti Sains Malaysia
WHO	World Health Organization

PENILAIAN *IN VITRO* AKTIVITI ANTIOKSIDAN DAN ANTIMIKROB SAMPEL MADU TUALANG DAN 5-HIDROKSIMETILFURFURAL TULEN

ABSTRAK

Madu tualang lama (ATH) kekal menjadi pilihan utama kerana manfaat nutrisinya yang tinggi, walaupun ramai yang tidak menyedari bahawa ia mengandungi kepekatan 5-hydroxymethylfurfural (HMF) yang tinggi. Kajian ini bertujuan untuk menilai sifat terapeutik sampel madu tualang (TH) dari pelbagai peringkat umur (FTH, ATH-24, ATH-48) yang mengandungi HMF semulajadi dibandingkan dengan HMF tulen (dos HMF rendah, sederhana and tinggi). Sifat antioksidan dinilai melalui ujian penghapusan radikal DPPH untuk mengira nilai IC_{50}. Seterusnya, aktiviti antimikrob sampel TH dinilai berdasarkan zon perencatan menggunakan kaedah penjerapan perigi agar sebaran. Menurut penemuan kajian, semua sampel TH menunjukkan aktiviti penghapusan radikal bebas yang lebih tinggi (ATH-48 paling tinggi) berbanding dengan kumpulan HMF tulen. Nilai IC_{50} yang dikira bagi sampel TH adalah lebih rendah berbanding nilai IC_{50} HMF tulen, menunjukkan potensi antioksidan yang lebih baik. Selain itu, sampel TH mempamerkan aktiviti antimikrob terhadap semua bakteria yang diuji. Aktiviti antimikrob paling tinggi, yang ditunjukkan melalui zon perencatan terbesar bagi setiap sampel TH, diperhatikan terhadap *S. aureus*. ATH-24 menunjukkan keberkesanan antimikrob tertinggi dengan zon perencatan yang lebih baik bagi kedua-dua bakteria Gram-positif dan Gram-negatif berbanding FTH dan ATH-48. Kumpulan HMF tulen menunjukkan kesan antioksidan yang bergantung kepada dos tanpa sebarang aktiviti antimikrob. Dengan mengambil kira semua data, ATH-24 dengan tahap HMF sederhana menunjukkan

sifat antioksidan dan antimikrob yang baik. Sebagai usaha memahami kelebihan pelbagai sampel TH yang mengandungi HMF semulajadi, kajian ini menunjukkan bahawa HMF mungkin meningkatkan kesan antioksidan secara sinergi dalam sampel TH tetapi bukan kesan antimikrob. Kajian ini menyumbang kepada pengembangan pengetahuan baru dalam bidang penyelidikan apiterapi.

***IN VITRO* EVALUATION OF THE ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TUALANG HONEY SAMPLES AND PURIFIED 5-HYDROXYMETHYLFURFURAL**

ABSTRACT

Aged tualang honey (ATH) remains a favoured choice due to its superior health benefits, despite many people being unaware that it contains elevated concentrations of 5-hydroxymethylfurfural (HMF). This study aimed to assess the therapeutic properties of TH samples of varying ages (FTH, ATH-24 and ATH-48) containing natural HMF in comparison with purified HMF (HMF low, medium and high doses). The antioxidant property was evaluated through a DPPH radical scavenging assay to calculate IC_{50}. Further, the antimicrobial activity of the TH samples was assessed based on the zone of inhibition by the agar well diffusion method. According to the findings, all TH samples demonstrated higher free radical scavenging activities (ATH-48 exhibited the highest) compared to purified HMF groups. The calculated IC_{50} values of TH samples were lower than the IC_{50} values of purified HMF, indicating their superior antioxidant potential. Apart from that, TH samples exhibited antimicrobial activity against all tested bacteria. The greatest antimicrobial activity, indicated by the largest inhibition zone of each TH sample, was observed against S. aureus. ATH-24 has the highest antimicrobial efficacy with better zones of inhibition for both Gram-positive and Gram-negative bacteria compared to FTH and ATH-48. Purified HMF groups demonstrated dose-dependent antioxidant effects without any antimicrobial activities. Taking all data together, ATH-24 with a medium level of HMF demonstrated good antioxidant and antimicrobial properties. As we uncover the advantages of TH samples containing natural HMF, these findings suggest that

HMF may synergistically enhance the antioxidant effects in TH samples but not the antimicrobial effects. This study contributes to expanding new insights in apitherapy research.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Human health is a significant concern in the contemporary world, as it is continually challenged by various factors such as lifestyle choices, environmental conditions, and microbial threats. The discovery of natural sources with potent therapeutic effects such as antioxidant and antimicrobial properties is significant to serve as complementary treatment. Oxidative stress has been implicated in the development of many chronic diseases (Forman and Zhang, 2021). Oxidative stress occurs when excess free radicals accumulate in the body, leading to degenerative diseases like heart disease, cancer, and diabetes (Reddy, 2023). Antioxidants are compounds that can neutralise these free radicals, preventing them from causing reactive damage to cellular lipids, enzymes, and DNA (Cleveland Clinic, 2021). Simultaneously, the rising antibiotic resistance among bacteria has created an urgent need for the discovery of alternative antibacterial agents. Antibiotic-resistant bacterial strains are becoming more common due to improper and excessive antibiotic use (Larsson and Flach, 2021), which makes it more challenging to treat infections.

Among the vast array of natural resources, honey stands out as a versatile and valuable resource that extends its significant role as a nutritional and medicinal oriented product. Honey is a naturally sweet and sticky biological fluid made by honeybees from plant nectar. It also contains small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals (Khalil *et al.*, 2011). One tablespoon of honey provides 64 calories, 17.2 grams (g) of sugar, and contains no fibre, fat, or protein. With an average pH level of 3.9, honey is slightly acidic, and research suggests that this acidity might inhibit bacterial growth (Nordqvist, 2023).

The medicinal properties of honey are determined by its chemical makeup, which varies depending on the botanical source of flora, geographical location, seasonal collection period and production techniques (Ayoub *et al*., 2023). According to numerous published research, honey possesses a wide range of pharmacological properties including antioxidant (Becerril-Sanchez *et al*., 2021) and antibacterial (Almasaudi, 2020) activities. Due to this reason, this current study focused on Malaysian tualang honey (TH) samples specifically fresh TH (FTH) and aged TH (ATH), which make them a suitable subject of interest for further investigation into their potential antioxidant and antimicrobial properties.

Tualang honey (TH) is a Malaysian wild polyfloral honey produced by the giant honeybee, *Apis dorsata* (Gengatharan & Gengatharan, 2023). The giant honeybees build nests hanging from the branches of one of the tallest tropical rainforest trees, the *Koompassia excelsa*, commonly known as tualang tree (Kamal *et al*., 2021). The colour of TH is dark brown, and it has a pH between 3.6 and 4.0 and a specific gravity of 1.34. The pH of TH is similar to that of manuka honey, and it is slightly more acidic than other native Malaysian honeys like kelulut and gelam (Azman *et al*., 2021). Interestingly, TH has more phenolic acids and flavonoids than Manuka and other local Malaysian honeys, and it is also more efficient against some Gram-negative bacteria (Ahmed and Othman, 2013). As well established, TH has been documented to exhibit antioxidant (Erejuwa *et al*., 2010), anticancer (Khalid *et al*., 2018), antimicrobial (Amir *et al*., 2020), anti-inflammatory (Silva *et al*., 2021) and antidiabetic (Aga *et al*., 2023) activities due to its high concentration of phenolic acids and flavonoids (Talebi *et al*., 2020). The availability of extensive scientific data on TH has sparked renewed interest in its usage as a dietary supplement and pharmaceutical product to enhance overall health and well-being.

It is important to acknowledge that the composition of TH undergoes changes over time, as evidenced by its acidity, moisture content and the concentration of bioactive components. These changes can affect both the nutritional values and medicinal properties of TH. In this context, FTH and ATH are distinct as FTH is new TH stored for a duration of up to six months while ATH is honey that has been stored for more than 12 months. In fact, ATH exhibits a darker physical appearance compared to FTH, which has a light amber-golden colour due to an increase in specific polyphenols or pigments (Sabireen *et al*., 2022). According to the previous investigations, the concentration of the neo-formed contaminant, 5-hydroxymethylfurfural (HMF) has increased in ATH that has been stored for a longer period. The same researchers have reported that ATH contains substantial levels of HMF (Khalil *et al*., 2011, Sabireen *et al*., 2022), frequently exceeding the 80 mg/kg limit specified by the Codex Alimentarius Commission (2001) for honey from tropical climates.

HMF, a cyclic aldehyde produced by the dehydration of fructose or glucose, primarily fructose, is an organic neo-formed hazardous contaminant (Sabireen *et al*., 2022). However, the HMF is often absent or present in very low amounts in FTH. The HMF concentration has been used as a key indicator of honey quality as well as to determine the freshness of honey, as it tends to increase due to ageing or during processing (Shapla *et al*., 2018).

In general, evidence on the properties and effects of ATH is sparse since most of the available studies solely focus on FTH. Even though ATH contains high secondary metabolites such as flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase and carotenoids that contribute to its potential medicinal and therapeutic properties, it remains unknown whether it has significant effects on the antioxidant and antimicrobial properties. Hence, the present study was conducted to further evaluate the antioxidant effects of TH samples containing natural HMF in comparison with purified HMF on a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Antioxidants are crucial as they can neutralise or inhibit free radicals and prevent oxidative damage to the cells (Reddy, 2023).

Furthermore, antimicrobial properties of TH samples and purified HMF were also investigated in this study through an agar well diffusion assay. The findings from this study may indicate that these test substances can disrupt the integrity of bacterial cell walls and inhibit essential protein and enzyme functions necessary for bacterial survival, thereby contributing to their antimicrobial efficacy (Al-Sayaghi *et al*., 2022). The evidence from this research could provide new scientific information on the therapeutic application of TH samples, particularly the ATH with higher HMF contents, as well as the effects of purified HMF. Overall, this study would highlight the benefits of TH associated with their storage period and thereby increase public awareness prior to use it.

1.2 Problem statement

It is worth mentioning that the majority of studies focus on exploring the wider benefits of FTH in general terms, rather than evaluating the therapeutic effects of ATH as well as the possible effects of natural HMF in TH. According to Khalil *et al*. (2011), all types of honey should be consumed within a year of manufacturing due to the production of HMF. Despite HMF has been linked to some negative effects such as mutagenic, carcinogenic and enzyme inhibiting effects, it on the other hand has been demonstrated to have a wide range of advantageous benefits in more recent extensive studies. These include antioxidative (Zhao *et al*., 2013), anti-allergic (Alizadeh *et al*., 2017), anti-inflammatory (Kong *et al*., 2019), anti-hypoxic (Choudhary *et al*., 2020), anti-sickling (Rajkumar *et al*., 2020), and anti-hyperuricemic (Shapla *et al*., 2018) effects.

Mohammad and Hassan (2021) stated that the HMF values in FTH ranged from 0.75 to 8.08 mg/kg. However, the HMF concentration in the ATH samples stored for one and two years exhibited initial HMF contents of 6.92 and 13.0 mg/kg, respectively. Samples stored for over two years exhibited the highest HMF values, reaching 109.61 mg/kg (Kishore *et al*., 2014) which exceeds the international standard limit (80 mg/kg) for tropical honey that is set by the Codex Alimentarius Commission (2001). This shows that FTH and ATH have different contents of HMF which tends to demonstrate various levels of antioxidant and antimicrobial effects.

HMF within TH may work synergistically with other bioactive compounds in TH such as flavonoids and phenolic acids to provide human health benefits. In contrast, the purified HMF has been reported to possess cytotoxic effects (Morales, 2008) and is less likely to be used to treat oxidative stress and bacterial infections. At high concentrations, it exhibits adverse effects causing cytotoxicity to mucous membranes, skin, and upper respiratory tract as well as demonstrating mutagenicity, carcinogenicity and cancer in both humans and animals (Farag *et al*., 2020).

Coupled with the fact that there is a lack of scientific information on ATH and HMF contents in honey, the general public remains unaware of the increasing HMF in TH over time. In addition, old folks in Malaysia are particularly very keen on consuming aged honey like ATH without knowing its possible impacts due to high HMF concentrations (Sabireen *et al*., 2022). They contend that older, darker-coloured honey contains more nutritive qualities than fresh, lighter-coloured honey. This scenario may result in the consumption of honey with a high HMF content while the functions and pharmacodynamic properties of HMF are still poorly understood. Moreover, there is no solid evidence to indicate whether it has beneficial or detrimental effects on human health.

1.3 Study objectives

1.3.1 General objective

The general objective of the present study was to evaluate the antioxidant and antimicrobial activities of TH samples and purified HMF *in vitro*.

1.3.2 Specific objectives

The specific objectives of this study are outlined as follows:

i. To examine the antioxidant effects of TH samples and purified HMF using a DPPH radical scavenging assay.

ii. To assess the antimicrobial activities of TH samples and purified HMF against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* using agar well diffusion assay.

iii. To compare the effects between natural HMF inside TH samples and purified HMF.

1.4 Hypothesis

1.4.1 Null Hypothesis

There was no significant difference in the antioxidant and antimicrobial activities between natural HMF inside TH samples and purified HMF.

1.4.2 Alternative Hypothesis

There was a significant difference in the antioxidant and antimicrobial activities between natural HMF inside TH samples and purified HMF.

1.5 Significance of study

The interest in evaluating the antioxidant and antimicrobial effects of TH samples, particularly the ATH arises from the fact that FTH possesses substantial therapeutic effects and most commonly serves as complementary treatment (Mohamed and Hamad Alfarisi, 2017). In contrast, the medicinal properties of ATH that contain elevated levels of HMF are still scarce. Apart from that, this experiment was conducted due to a poor understanding of the relative comparison between natural HMF within TH and purified HMF. Therefore, this study was conducted with the main aim of gathering the therapeutic effects of antioxidant and antimicrobial properties of TH samples containing natural HMF and the purified HMF *in vitro*.

The findings of this study can contribute greatly to society because TH has been used since ancient times due to its various health benefits. Despite medical advances achieved today, TH remains an integral part of traditional medicine in a particular demographic region for centuries (Khalid *et al*., 2018). The health benefits of TH have long been gaining increased attention and popularity, as evidenced by numerous documented studies. Hence, the conduct of this present study was crucial to allow the discovery of natural resources with potential therapeutic effects specifically antioxidant and antimicrobial properties.

Finally, studying purified HMF can elucidate to what extent HMF participates in the integrated antioxidant and antimicrobial properties of TH. This could lead to a deeper understanding of how processing and storage conditions impact the health benefits of TH and hence contributing valuable knowledge for the scientific community.

CHAPTER 2

LITERATURE REVIEW

2.1 Tualang honey (TH)

2.1.1 Overview of TH

TH of the Agromas® brand is a multifloral jungle honey, produced by the Asian wild rock bee *Apis dorsata* that builds its honeycombs on the high branches of tualang trees (Bujor, 2015). The name TH comes from the tualang tree scientifically named *Koompassia excelsa*, a large tree found in lowland tropical rainforests in Malaysia, Kalimantan and Thailand (Amanda, 2024). A single tualang tree can host over 100 nests, producing approximately 450 kilograms (around 1000 pounds) of honey (Mohamed *et al*., 2010). In Malaysia, tualang trees are abundantly found in the northeastern region, in the state of Kedah (Ahmed and Othman, 2013). TH is primarily collected in the areas of Padang Terap, Sik, Baling, and Gunung Jerai of Kedah. The harvesting of TH occurs exclusively during the peak flowering season, which spans from December to April (Nawawi, 2015).

The giant bees, known as *Apis dorsata*, have a strong preference for these types of trees, as they provide an ideal height for constructing their nests safely away from the reach of both animals and humans. This relationship fosters a beneficial synergy, where the trees serve as a habitat for the bees, while the bees, in turn, offer protection to the trees from logging activities. The bee hive can be up to 6 feet high and each hive may have an average of 30,000 bees (Bujor, 2015). Additionally, the tualang tree is noted for being one of the tallest tropical trees, with a potential maximum height of 250 feet (Amran *et al*., 2020). The honey reaches the appropriate level of maturity for harvesting, typically during the moonless nights of February and

March. During this period, honey hunters, armed with smoldering torches ascend the tualang trees and strike against the branches that support the nests. This action produces a cascade of sparks, and as these embers fall to the ground, the disturbed and furious bees emerge to chase after them. The bees, however, become confused and linger on the ground until morning, leaving their nests vulnerable. Consequently, the honey hunters can collect honey, with a single tree yielding approximately 1,000 pounds (Bujor, 2015).

TH Agromas® has been a product of the FAMA, a marketing organisation operating under the Ministry of Agriculture and Food Industry, Malaysia since 1996. According to a FAMA officer in Padang Terap, Pedu Lake Forest Reserve and Ulu Muda are the treasure trove of tualang trees and TH colonies (Bernama, 2018). As a result of its distinctive aroma and flavour, the honey harvested from this area is of the highest quality and renowned throughout the entire nation and the world (FAMA Kuala Nerang Kedah, 2023). In accordance with the Food Regulation 1985 (update 2023), Ministry of Health (MOH), Malaysia, the harvested honey is sent to the Honey Processing Centre, FAMA Kuala Nerang, Kedah, for further analysis and processing.

Typically, TH undergoes multiple phases of processing at the Honey Processing Centre, FAMA such as quality assessment, dehydration, packaging and labelling. Upon arrival at the centre, TH is analysed for its water (moisture) and sugar levels, along with aroma and flavour to verify the product’s authenticity. The approved TH must contain an average sugar content of approximately 75% and a water content of about 20%, in accordance with the standards established by the MOH. The TH will subsequently undergo a weighing and filtration process to eliminate any foreign particles. After this step, it will be processed through evaporation, which lasts between eight and 16 hours, aiming to remove approximately 3-5% of excess water. Evaporation plays a crucial role in regulating the water content to a specific level, helping to avert the fermentation and souring of TH (Bernama, 2018). Ultimately, TH will be subjected to a second filtration process prior to being bottled, weighed and packaged under the Agromas® brand for distribution throughout Malaysia (FAMA Kuala Nerang Kedah, 2023). The procedures for processing TH by FAMA are illustrated in Figure 2.1 and Figure 2.2.

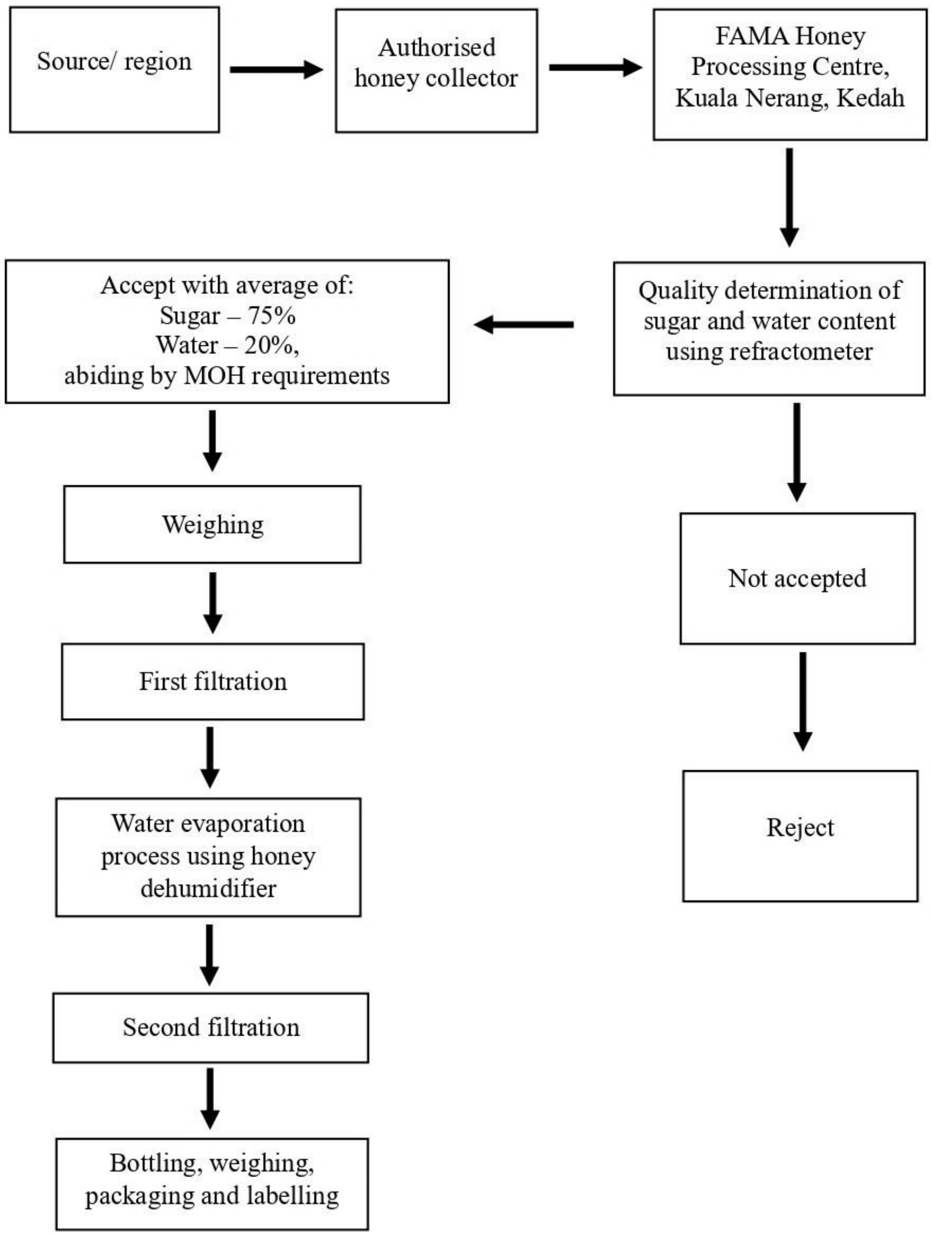

Figure 2.1 TH Agromas® processing chart.

Figure 2.2 Visual depicting TH Agromas® processing at the Honey Processing Centre and available for purchase at the House of Honey, FAMA Kuala Nerang, Kedah.

A: Refractometer used for measuring sugar and water content in TH. **B**: Honey processing centre. **C**: Presentation of the TH processing methods. **D**: TH in the oven. **E**: TH in honey dehumidifier. **F** and **G**: The bottled and packaged TH is now available for purchase at the House of Honey (FAMA Kuala Nerang Kedah, 2023).

2.1.2 Physicochemical characteristics

TH is characterized by its distinctive physical properties, which feature a deep brown colour with high intensity. TH possesses a specific gravity of 1.34 and has a pH level ranging from 3.60 to 4.00 (Azman *et al*., 2021). This low pH is comparable to that of manuka honey, which measures between 3.2 and 4.21. In fact, TH is more acidic than other types of Malaysian honey, including kelulut hitam, kelulut putih, and gelam (Ahmed and Othman, 2013). In addition, TH largely adheres to the established guidelines set forth by the two primary regulatory frameworks, namely the European Honey Legislation and the Codex Alimentarius Standards. TH that exhibits a moisture content exceeding 20% violates the specified regulations. However, it is worth noting that honey sourced from tropical regions like Malaysia exhibits elevated moisture levels likely as a result of the year-round rainy climate (Al-Kafaween *et al*., 2023). Meanwhile, the electrical conductivity of honey serves as a means to ascertain its botanical source and purity. The electrical conductivity of TH falls in a broad range of 0.74–1.51 mS/cm (Kamal *et al*., 2021).

Amongst the various types of Malaysian honey, TH is the richest in phenolic acids and flavonoid compounds (Chua *et al*., 2012). Phenolic acids refer to compounds that feature a phenolic ring structure, for example gallic, coumaric, syringic and benzoic. These phenolic substances that are present in TH could be represented as medicines for the treatment of cardiovascular diseases (Al-Kafaween *et al*., 2023). Besides, flavonoids are primarily water-soluble natural compounds with a low molecular weight, including catechin, quercetin, kaempferol and luteolin (Azman *et al*., 2021). They play a vital role in diminishing oxidative stress and are essential for the antioxidant properties of TH.

Apart from that, the fractionation of TH found that 65% of the component in this honey is sugar, comprising monosaccharides such as glucose and fructose (Moniruzzaman *et. al*., 2013). According to Chua and Adnan (2014), glucose is the most abundant sugar in TH (47.13%), followed by fructose (41.73%), then maltose (4.49%), and sucrose (1.02%). The protein content of TH is reported as 3.6 to 6.6 g/kg with potassium (K) having the highest concentration followed by sodium (Na), calcium (Ca), and magnesium (Mg) (Moniruzzaman *et al*., 2013). Sakika *et al*. (2022) utilised gas chromatography-mass spectrometry to profile the major volatile compounds found in TH such as oxygenated compounds, hydrocarbons and heterocyclic compounds.

2.1.3 Medicinal properties of TH

The health benefits of TH are primarily attributed to its rich content of phenolic compounds, particularly polyphenols like phenolic acids and flavonoids. These compounds which are found in significant quantities within TH act as free radical scavengers, peroxy-radical scavengers, and metal chelators (Gengatharan & Gengatharan, 2023). The dark brown colour of TH is associated with a significant ferric-reducing power, which shows a positive correlation with elevated levels of phenolic acids and flavonoids (Talebi *et al*., 2020). TH has demonstrated a significant ability to scavenge free radicals, potentially aiding in the prevention of cancer development. A study reported that TH effectively induced apoptosis in breast cancer cell lines (MDA-MB-231 and MCF7) as well as in cervical cancer cells (HeLa) depending on the dosage and duration of exposure (Fauzi *et al*., 2011). Apart from this, Ahmed and Othman (2017) showed a significant decrease in the volume of palpable tumours, shrinking from 1.47 to 0.26 cm^3 by treating cancer-induced female Sprague-Dawley rats with 0.2 g/Kg of TH.

Further research has revealed that TH has wound-healing properties. A clinical study demonstrated that topical application of TH on burn wounds contaminated *with P. aeruginosa* and *A. baumannii* provided a faster healing rate than chitosan gel or hydrofibre silver treatment (Sukur *et al*. 2011). The result of this experiment clearly indicates that burn wounds treated with TH exhibited significantly greater *in vivo* wound contraction compared to those dressed with hydrofibre silver and hydrofibre dressing (Mohamed and Hamad Alfarisi, 2017). The effectiveness of TH as a dressing material can be attributed to its antimicrobial properties. The antimicrobial effects are attributed to the combination of high osmolarity, low pH levels and the presence of catalase, which prevents the proliferation of microorganisms (Wilkinson and Cavanagh, 2005).

Moreover, TH also has the potential to be a natural pain reliever due to its antinociceptive activity. According to Kishore *et al*. (2011), the antinociceptive effects could be due to its antioxidant properties, which are comparable to 53.06 ± 0.41 mg of ascorbic acid per gram of TH. Similarly, the antioxidants found in TH may have interfered with nociceptive transmission by engaging with glutamate receptors in the central nervous system (Azman *et al*., 2021). In a clinical study, taking 4 mL of TH three times per day for a week, combined with sultamicillin antibiotic, has been shown to significantly enhance early postoperative pain relief in patients who underwent tonsillectomy (Abdullah *et al*., 2015).

2.2 5-Hydroxymethylfurfural (HMF)

2.2.1 Overview of HMF

HMF is a six-carbon cyclic organic molecule characterized by the presence of both aldehyde and hydroxymethyl (alcohol) functional groups as demonstrated in Figure 2.3. It is highly soluble in water and is known for its volatility, with melting and boiling points recorded at 31.5°C and 115°C, respectively (Choudhary et al., 2020). The level of HMF produced directly depends on factors such as pH, temperature and specific composition of the food products. Hence the food industry utilises HMF as an indicator of quality and potential adulteration in various items, including coffee, juices, milk, sauces, honey, and cereals (Lee *et al*., 2019).

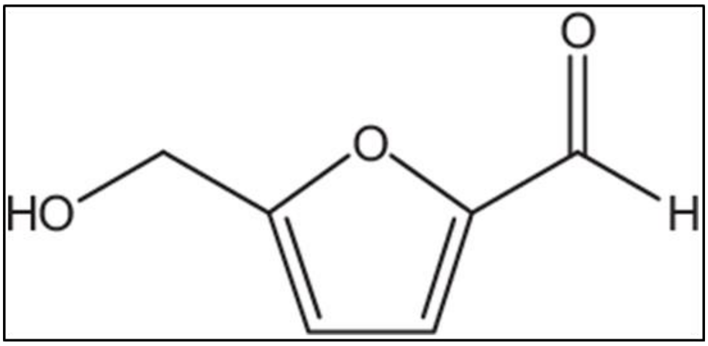

Figure 2.3 Structure of 5-Hydroxymethylfurfural (HMF).

HMF is regarded as a crucial intermediate formed through two specific processes. The first is the Maillard reaction, which involves the breakdown of 3-deoxyglucosone, and the second pathway is caramelisation, which involves the dehydration of sugars in an acidic environment (Fallico *et al*., 2008). The efficiency of HMF formation is directly influenced by water activity, cation and amino acid concentrations, type of sugar as well as temperature and pH of the medium (Gokmen and Morales, 2014). Notably, elevated fructose levels, the presence of sodium and an acidic environment favour the formation of HMF. In contrast, increased water level hinders the formation of HMF by inhibiting sugar dehydration (Lee *et al*., 2019). The detailed mechanism of HMF formation is illustrated in Figure 2.4.

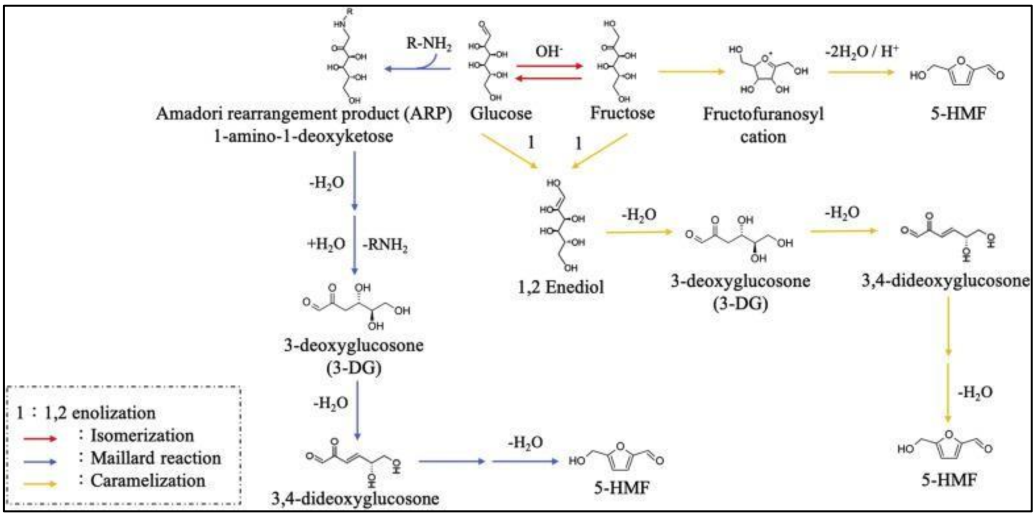

Figure 2.4 The biosynthesis of HMF (Zhang & An, 2017)

2.2.2 Concentration of HMF in TH

HMF typically develops in honey as a result of sugar dehydration through a non-enzymatic browning reaction during food processing and long-term storage. The presence of simple sugars such as fructose and glucose, along with various acids and minerals found in honey, can significantly boost the production of HMF (Shapla *et al*., 2018). The concentration of HMF is commonly acknowledged as an important indicator of honey freshness (Surma *et al*., 2023). In fresh honeys, HMF is usually absent or found in minimal quantities, whereas its levels tend to increase with processing. Previous studies have reported that TH kept at low temperatures or under optimal conditions tends to have low or minimal HMF concentrations. In contrast, TH that has been aged or stored at higher or moderate temperatures exhibits elevated HMF concentrations (Shapla *et al*. 2018).

A previous research of ATH samples stored for over 4 years revealed that the levels of the notably neo-formed contaminant, HMF were significantly higher than the internationally recommended limit of 80 mg/kg (Sabireen *et al*., 2022). Moniruzzaman *et al*. (2013) reported that the mean HMF concentration in TH stored for 2 months at 4–5°C to be 35.98 mg/kg. Whereas Khalil *et al*. (2010) discovered that TH kept at 25–30°C for more than a year (considered ATH), resulting in very high levels of HMF ranging from 118.47–1139.95 mg/kg. Another study also reported that besides storage duration, the HMF formation was intensified at low pH. For instance, ATH with a pH of 3.28, exhibits a significantly greater concentration of HMF at 1426 mg/kg, compared to that of FTH, which has a pH of 3.59 and contains only 27 mg/kg of HMF (Sabireen *et al*., 2022). Therefore, the concentration of HMF serves as a marker not only for the freshness of honey but also for the storage duration and conditions under which TH has been stored.

2.2.3 Beneficial properties of HMF

A variety of research indicates that HMF possesses several beneficial effects on human health. Primarily, HMF acts as a potent antioxidant. By scavenging free radicals, the HMF actively eliminates reactive oxygen species which cause cellular damage (Lee *et al*., 2019). HMF also possesses a significant protective effect on hepatocytes against hydrogen peroxide (H_2O_2)-induced oxidative stress. Under such conditions, hepatocytes are preserved from experiencing morphological alterations including chromatin condensation, wrinkling and nuclei splitting (Shapla *et al*., 2018). In addition, HMF enhances the effectiveness of antigen-antibody crosslinking and supports antibody-receptor binding. It also blocks the influx of calcium (Ca^{2+}) into IgE-sensitized bovine serum albumin stimulated RBL-2H3 cells. Hence, HMF has been shown to exhibit anti-allergic activity by inhibiting the release of histamine and disrupting Ca^{2+} signaling (Li, 2009). Furthermore, HMF serves as an effective anti-sickling agent. In a study conducted on transgenic (Tg) sickle mice, HMF given orally in low doses is absorbed into the bloodstream via the gastrointestinal tract. Once in circulation, HMF can enter erythrocytes and form a stable Schiff-base adduct with the N terminal αV11 nitrogen of HbS in a symmetrical fashion. Following the formation, HMF causes an allosteric leftward shift in the oxygen equilibrium curve, effectively inhibiting sickling of erythrocytes (Abdulmalik *et al*., 2005).

2.2.4 Disadvantages of HMF

Despite the numerous beneficial effects of HMF, it is also reported to possess disadvantages. HMF and its derivatives have been shown to exhibit negative impacts on human health including genotoxic, mutagenic, carcinogenic, DNA-damaging, organotoxic and enzyme-inhibitory effects. A study by Lee *et al*. (1995), has demonstrated that HMF is an indirect mutagen, as it transforms into a reactive metabolite known as 5-sulfoxy-methylfurfural (SMF), which exhibits mutagenic properties against *S. typhimurium* TA104. Another study on FVB/N (FVB) mice expressing hSULT1A1/1A2 showed that HMF possesses genotoxic properties. The mice were orally administered single doses of 900 or 1300 mg/kg of HMF exhibited significant DNA damage to their renal cells (Hoie *et al*., 2015). Moreover, HMF induces neoplastic changes in multiple organs such as the colon and skin. Evidence from a previous study revealed that 45% of F344 female rats given HMF at a dosage of 250 mg/kg twice per day by oral gavaging developed a large intestinal aberrant crypt focus (ACF) by day 30 (Shapla *et al*., 2018). HMF also induces papilloma on their skin upon topical application. At elevated concentrations, HMF exhibits cytotoxic properties, leading to irritation of mucous membranes, skin, eyes and the upper respiratory tract (Morales, 2008).

2.3 Antioxidant properties

2.3.1 Antioxidant properties of TH

An antioxidant substance plays a crucial role in significantly mitigating the oxidation of the oxidisable substrate for the survival of all organisms. Humans own highly complex antioxidant systems, both enzymatic and non-enzymatic, that function collaboratively to protect cells and organ systems from damage caused by free radicals. The most efficient enzymatic antioxidants contain glutathione peroxidase, catalase and superoxide dismutase. Non-enzymatic antioxidants include vitamin E and C, thiol antioxidants, melatonin, carotenoids and natural flavonoids. However, the body is unable to generate certain essential antioxidants on its own. Therefore, it is crucial to obtain them from external sources like food or supplements. Recent studies indicate that plant-based antioxidants with free-radical scavenging properties may play a significant role as therapeutic agents in treating various diseases linked to oxidative stress (Sen *et al*., 2010). These effective antioxidants can be readily absorbed, neutralise free radicals and bind to redox metals at levels that are appropriate for physiological conditions.

Since several plant products are rich in antioxidants and micronutrients, it is possible that dietary antioxidant supplementations protect against the reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated disease development. Therefore, natural-based antioxidant supplementation has become an increasingly popular practice to maintain optimal body function. TH is a natural product that offers a promising ability to counteract the overproduction of harmful free radicals. The free radicals can contribute to the development of chronic and degenerative diseases such as cardiovascular disease, carcinogenesis, diabetes mellitus and stroke (Phaniendra, Jestadi and Periyasamy, 2014). The major factors that contribute to the excessive generation of ROS and RNS are exposure to cigarette smoking, alcohol consumption, radiation and environmental toxins.

The antioxidant activity of TH can be mainly attributed to the phenolic compounds in the honey, including flavonoids and phenolic acids which act as free radical scavengers, peroxy-radical scavengers and metal chelators. There are more than 150 polyphenolic compounds comprised of both enzymatic and non-enzymatic substances. Radical scavengers in honey can effectively interact with and neutralise peroxide radicals, halting the peroxidation chain reaction that enhances the quality and stability of food products. Chew *et al*. (2018) found that the process of fractioning TH after acid hydrolysis significantly enhanced its bioactive properties particularly in terms of free radical scavenging effects. Specifically, TH fractionated with ethyl acetate exhibited an increase in frequency of scavenging ability ranging from 35 to 165 times. A study conducted by Erejuwa *et al*. (2010) demonstrated that a combination of TH with either glibenclamide or metformin provided an additional antioxidant effect on the kidneys of diabetic rats. The effect was achieved by increasing the expression of the catalase gene while simultaneously decreasing the expression of the glutathione peroxidase gene.

2.3.2 Method of antioxidant determination

Chemical tests for assessing antioxidant properties can be broadly categorized into two main groups: hydrogen atom transfer (HAT) and single electron transfer (SET) reaction-based methods. Despite differences in kinetics and reaction stages, both mechanisms produce the same result. HAT tests assess an antioxidant's ability to neutralise free radicals by donating a hydrogen atom. Examples of HAT-based tests are oxygen radical absorption capacity (ORAC), total peroxyl radical trapping antioxidant parameter (TRAP), and total oxyradical scavenging capacity

(TOSC) assay. Conversely, SET tests measure the effectiveness of an antioxidant to transfer an electron to neutralise free radicals, carbonyl groups, and metallic ions. Common examples of these electron transfer-based assays include 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric reducing antioxidant capacity (CUPRAC), Folin–Ciocalteu test (FC) and ferric reduction of antioxidant power (FRAP) (Munteanu and Apetrei, 2021).

Radical scavenging-based assay, DPPH is the most popular and widely used spectrophotometric technique for accessing the antioxidant activity of beverages, foods and plant extracts. This technique is highly valued for its ability to directly engage with antioxidant compounds, as well as due to their sensitivity, ease of use and reproducibility. The DPPH radical was identified a century ago by researchers Goldschmidt and Renn in 1922 (Foti, 2015). The chemical structure of the DPPH radical is illustrated in Figure 2.5. The single electron of the nitrogen atom in DPPH is converted to the corresponding hydrazine by accepting a hydrogen atom from the antioxidants. Additionally, the DPPH radical is characterised by its stable and intense colour. These two properties of the radical allow its solution to be widely used for assessing the antioxidant potential of various substances.

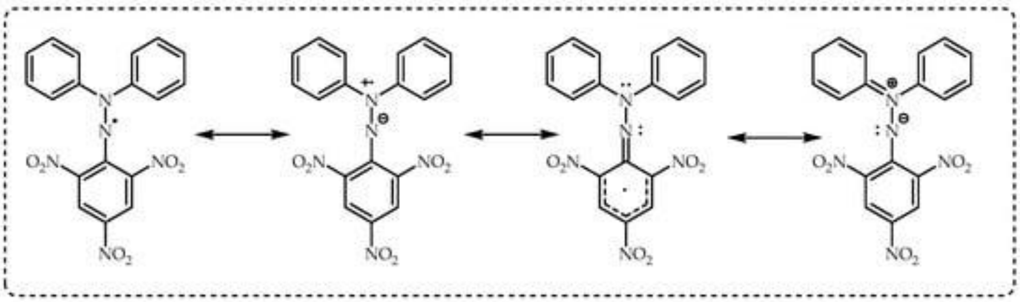

Figure 2.5 The chemical structures of a DPPH (Gulcin & Alwasel, 2023).

Phenolic compounds in TH are the most reactive compounds that react effectively with DPPH radicals by electron transfer or by donating hydrogen atoms.

When the free radical DPPH solution is mixed with a TH sample capable of donating a hydrogen atom, its violet colour disappears, resulting in the reduced form of the DPPH radical (DPPH-H) (Yapıcı *et al*., 2021). The formation of hydrazine (DPPH-H) induces the disappearance of the visible band as the colour of the solution changes from violet to pale yellow as a result of radical reduction. This reduction occurs when the unpaired electron of the DPPH radical pairs with hydrogen atoms from an antioxidant, which are H donors. The intensity of colour observed in this reaction can be conveniently measured using UV-visible spectroscopy. This technique is widely employed to evaluate the antioxidant capacity of antioxidant molecules particularly those derived from plants and rich in phenolic compounds (Xie and Schaich, 2014).

The antioxidant activity of TH is assessed through the percentage inhibition of DPPH free radicals. The research findings represent the antioxidant activity in terms of IC_{50}, which means the concentration of antioxidant substances required to achieve a 50% inhibition of DPPH.

2.4 Antimicrobial properties

2.4.1 Antimicrobial activity of TH

Antimicrobials are substances intended to either suppress the growth or kill microorganisms such as bacteria, viruses, fungi and protozoa (Soares *et al*., 2012) that can lead to severe infections and illness in humans, animals and plants. Antimicrobial agents function through various mechanisms, including the disruption of cell membranes, inhibition of cell wall formation, interference with protein or DNA synthesis and blocking of essential metabolic pathways (Reygaert, 2018). They are crucial in both medical and agricultural settings to control infections and microbial contamination.

TH has been found to exhibit bactericidal and bacteriostatic activities against various species of wound and enteric bacteria. The antimicrobial effects of TH are primarily attributed to the high osmolarity, acidity and peroxidase activity (Ahmed & Othman, 2013). Additionally, TH contains non-peroxidase compounds including phenolic acids, flavonoids and lysozymes which also contribute to its antimicrobial effects (Alnaqdy *et al*., 2005). Tan *et al*. (2009) demonstrated that the minimum inhibitory concentration (MIC) for TH ranged from 8.75% to 25% (w/v) against bacteria such as *Streptococcus pyogenes,* local clinical isolates of coagulase-negative Staphylococci, *Methicillin-resistant Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, local clinical isolates of *Shigella flexneri* and *Escherichia coli*.

A study showed that certain antibacterial proteins or peptides found in TH can alter the transmembrane potential, impacting the permeability of bacterial membranes. Apart from that, the protein component in TH may obstruct the production of bacterial proteins or stimulate the expression of enzymatic activity like