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DETERMINATION OF ANTIOXIDANT, NITRIC OXIDE SCAVENGING ACTIVITY, TOTAL PHENOLIC AND FLAVONOID CONTENT FROM PROPOLIS OF *GENIOTRIGONA THORACICA*

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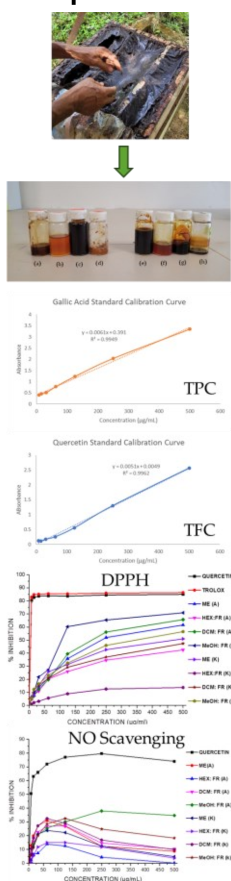
Graphical abstract



Abstract

Propolis, a substance stingless bees produce for nest construction and protection, has attracted great interest because of its potential biological effects. This study aims to determine the antioxidant activity, nitric oxide (NO) scavenging activity, total phenolic (TPC) and flavonoid (TFC) content of *Geniotrigona thoracica (G. thoracica)* propolis from two locations, namely Ketereh (K) and Besut Apiary (A). Propolis undergoes maceration with methanol for three days to form methanol extract (ME) and fractionation to afford hexane (HEX: FR), dichloromethane (DCM: FR) and methanol fractions (MeOH: FR) for both locations. TPC and TFC contents were calculated as gallic acid and quercetin equivalents. MeOH: FR (A) has 179.13 ± 0.03 mg/mL GAE for TPC and 375.05 ± 0.05 mg/mL QE for TFC. MeOH: FR (A) has an IC_{50} value of 118.11 µg/mL for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 27.68 µg/mL for 2,2'-casino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Furthermore, MeOH: FR (A) has 32.77 ± 0.33 µM (Fe^{2+}/mL sample) for ferric-reducing antioxidant power (FRAP) activity and 37.94 ± 0.01% for NO removal. Due to its strong antioxidant and NO scavenging ability, methanol fractions can be considered a valuable source to isolate bioactive compounds that promote anti-inflammatory responses and contribute to the prevention of human diseases.

Keywords: Propolis, *Geniotrigona thoracica*, antioxidants, nitric oxide scavenging activity

Abstrak

Propolis, suatu bahan yang dihasilkan oleh lebah kelulut untuk pembinaan sarang, telah menarik minat kerana beberapa dekad kerana potensi kesan antioksidannya. Kajian ini bertujuan untuk menentukan aktiviti antioksidan, aktiviti penghapusan nitrik oksida (NO), kandungan fenolik total (TPC) dan flavonoid (TFC) propolis *Geniotrigona thoracica (G. thoracica)* daripada dua lokasi, iaitu Ketereh (K) dan Besut Apiary (A). Propolis menjalani proses perendaman dengan metanol selama tiga hari untuk membentuk ekstrak methanol (ME) dan proses lanjut untuk membentuk pecahan daripada heksana (HEX: FR), diklorometana (DCM: FR), dan methanol (MeOH: FR). Kandungan TPC dan TFC dikira sebagai

setara asid gallic dan quercetin. MeOH: FR (A) mempunyai 179.13 ± 0.03 mg/mL GAE untuk TPC dan 375.05 ± 0.05 mg/mL QE untuk TFC. MeOH: FR (A) mempunyai nilai IC_{50} 118.11 µg/mL untuk 2,2-diphenyl-1-picrylhydrazyl (DPPH) dan 27.68 µg/mL 2,2'-casino-bis (3-ethylbenzothiazoline- asid sulfonik) (ABTS) dengan Tambahan pula, MeOH: FR (A) mempunyai 32.77 ± 0.33 µM (Fe^{2+}/mL sampel) untuk aktiviti kuasa antioksidan pengurangan ion besi (FRAP) dan 37.94 ± 0.01% untuk penyingkiran NO. Disebabkan oleh keupayaan antioksidan dan penyingkiran NO yang kuat, pecahan metanol boleh dianggap sebagai sumber berharga dan semula jadi yang mempromosikan tindak balas anti-radang dan menyumbang kepada pencegahan penyakit manusia.

Kata kunci: Propolis, *Geniotrigona thoracica*, antioksidan, aktiviti penghapusan nitrik oksida

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1.0 INTRODUCTION

About 600 species of stingless bees, belonging to the Meliponini tribe, are found worldwide in tropical and subtropical regions (Rasmussen and Cameron 2007) [1]. There are 78 species of stingless bees known to exist in Malaysian forests and rural regions [2]. In Malaysia, several stingless bee species are valued commercially for their honey, such as *Geniotrigona thoracica (G. thoracica), Lepidotrigona terminata (L. terminata), Heterotrigona itama (H. itama), Tetragonula laeviceps (T. laeviceps), Tetrigona apicalis (T. apicalis), Tetragonula fuscobalteata (T. fuscobalteata), Homotrigona fimbriata (H. fimbriata) and Tetrigona Binghami (T. binghami) [2][3]*. Propolis, often known as bee glue, is a combination of sticky substances that may be found in stingless bee mandibular secretion, flowers, buds, and leaves [1,4]. Due to the lipid content of over 45%, it is waterproof, and it can restrict the growth of bacteria such as *Escherichia coli (E. coli*), *Pseudomonas aeruginous (P. aeruginous), Staphylococcus aureus (S. aureus)* and *Bacillus subtilis (B. subtilis)* [5]. Propolis is also reported to have several bioactivities such as antibacterial [6], antioxidant [7], antidiabetic [8], antifungal [9] and anticancer [10]. Owing to these bioactivities, propolis has been used as a component of health foods, as well as a significant treatment in conventional medicine, contemporary biomedicine, and natural cosmetics [11].

According to Shittu *et al*. (2015), stingless bees collect propolis from plants surrounding the hive. Propolis is a material that resembles gum, and its colour ranges from dark brown to soft yellow [12]. Bees coat the inside walls of their hives with a thin sticky layer of propolis. It is used to seal cracks and holes, fix combs, strengthen the comb's weak edges, and weatherproof or make it simpler to protect the hive's entrance. In their hives, propolis serves as a protective antibacterial agent and is also utilized as a building element. Since propolis can cover invaders who have been killed by bees but are unable to leave the hive, it also serves as an "embalming substance". Because of its lipophilic qualities, propolis is brittle and stiff in the cold but pliable, mushy, and extremely sticky in the heat [11]. Propolis typically comprises 5% pollen grains, microelements, and vitamins, 5–10% essential oils, 30–40% waxes and 50–60% resins and balms [7]. Propolis typically contains many bioactive substances, including sugars, alcohol, terpenoids, flavonoids, fatty acids, aliphatic, aromatic acids, and esters. By using gas chromatography-mass spectroscopy (GC-MS) on *G. thoracica* propolis from Malaysia, it was revealed that several compounds were present, such as benzoic acid, phenol, β-amyrenol, nootkatone, octadecanoic acid, myristic, palmitic, linoleic, cycloeucalenol, friedelany-al, Δ-cadinene, resorcinol hydroginkgol, and trimethylsilyl ester [14], [15].

Around the world, propolis has been shown to include 180 distinct chemicals, most of which are polyphenols. These include phenolic acids, flavonoids, esters, ketones, and phenolic aldehydes. The quantity of polyphenols from propolis is frequently used as a marker to analyze the quality of the propolis. Capillary zone electrophoresis was used to identify 12 distinct flavonoids (quercetin, galangin, luteolin, rutin, naringenin, chrysin, myricetin, catechin, pinocembrin, acacetin, apigenin and kaempferol), one stilbene derivative (resveratrol) and two phenolic acids (caffeic acid and cinnamic acid), in propolis extracts. The resinous smell of propolis is caused by terpenes, which are also occasionally used as a criterion to differentiate between regular and premium propolis. Isocupressic acid, geraniol, limonene, lupeol, a-amyrin, and β-amyrin are some of the terpene chemicals found in propolis. Additionally, propolis contains hydrocarbons (alkenes, alkanes, diesters, monoesters, aromatic esters, and alkadienes), biometabolites (nucleic acids, amino acids, sugars, and lipids), beneficial minerals (zinc, calcium, magnesium, potassium, sodium, manganese, copper, and iron) and essential vitamins (B1, B2, B6, C, and E). Propolis also contains several other enzymes, including beta-amylase, acid phosphatase, succinic dehydrogenase, glucose 6-phosphatase, and adenosine triphosphatase [16].

Any substance with one or more unpaired electrons is considered a free radical, with one of those electrons in the orbital of an atom or molecule.

Reactive oxygen species (ROS) and free radicals, including superoxide anion ($O2^-$), hydroxyl ions (HO^-) and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species (RNS), notably gaseous molecule such as nitric oxide (NO), may be created by cellular metabolism as well as exposure to external factors including UV light, poisons, and drugs. Numerous human disorders, including cancer, diabetes, heart disease, neurodegenerative or cardiovascular disease, and atherosclerosis, may be caused by oxidative stress. Any substance that delays or stops oxidative damage to a target molecule is considered an antioxidant. Antioxidant substances that scavenge free radicals like hydroperoxide, lipid peroxyl or peroxide include polyphenols, phenolic acid, and flavonoids. This inhibits the oxidative process, which results in degenerative mechanisms [7].

Nitric oxide (NO) is a water-soluble gaseous molecule that can easily permeate across the cell membranes. The free radical structure, making it have an extra electron, allowing it to be highly reactive. NO serves as a mediator to signal the molecule in the cellular processes such as neurotransmission, immune response and regulation of gene transcription [17]. NO can act as anti-inflammatory and pro-inflammatory depending on the conditions and type of cells. KB nuclear factor (NF-κB), cyclooxygenase 2, and pre-inflammatory cytokines can all be stimulated and regulated by low levels of NO generated by endothelial nitric oxide (eNOS). High eNOS levels in kidney tissue and during renal ischemia-reperfusion, however, can be crucial in lowering inflammation, oxidative stress, and damage to renal tissue. However, elevated levels of the isoform of inducible nitric oxide synthase (iNOS) isoform worsen inflammation and damage [18].

Geographical location and botanical origin impact propolis's chemical composition because different plant species' resins can contain other compounds. Several variables influence the chemical composition of propolis, including the harvest season, the vegetation, and the sample collection site [15]. The objectives of this research were to determine the total amount of phenolic and flavonoid content and evaluate the antioxidant (DPPH, ABTS and FRAP) and NO scavenging capabilities of propolis methanolic extracts and their fractions from *G. thoracica* propolis that was gathered from two locations: Besut Apiary (A) and Ketereh (K).

2.0 METHODOLOGY

2.1 Chemicals

All chemicals and reagents used were freshly prepared and of analytical grade. Methanol, hexane, dichloromethane, DPPH, sodium carbonate (Na_2SO_4), Aluminum chloride hexahydrate ($AlCl_3.6H_2O$), Folin–Ciocalteu reagent, potassium acetate (CH_3CO_2K), Dimethyl sulfoxide (DMSO), potassium persulfate, ABTS salt, sodium acetate trihydrate, glacial acetic acid, Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), hydrochloric acid (HCl), ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), sodium nitroprusside (SNP), phosphate buffer, , sulphanilic acid, naphthyl ethylene diamine dihydrochloride (NEDD), trolox, quercetin and gallic acid were used in this study.

2.2 Propolis Samples

G. thoracica propolis samples were gathered from two sites, Besut Apiary (A) and Ketereh (K). The vegetation of the sampling locations has been observed in Table 1, and the propolis collection harmed no endangered or protected species.

Table 1 Sample localities, sample codes, and type of vegetation surrounding the hive

Sampling Localities	Sample code	Type of vegetation
Besut Apiary in Universiti Sultan Zainal Abdin (UniSZA), Besut Campus	A ME (A) HEX: FR (A) DCM: FR (A) MeOH: FR (A)	*Melaleuca viridiflora (Gelam Merah), Melaleuca cajuputi (Gelam putih), Acacia auriculiformis (Akasia kuning), Acacia mangium (Akasia puteh) and Baeckea frutescens (Cucur atap)*
Honeyzull Madu Kelulut Asli RZ Garden, Ketereh	K ME (K) HEX: FR (K) DCM: FR (K) MeOH: FR (K)	*Turnera ulmifolia (Bunga pukul lapan kuning), Mangnifera Indica (Pokok Mangga epal), Melastoma imbricatum (Senduduk putih), Xanthostemon chrysanthus (Yellow penda), Citharexylum spinosum (Bunga mayang sari), calliandra*

2.3 Propolis Extraction and Fractionation *Via* Maceration

The maceration method was used to extract the propolis from two localities: Besut Apiary and Ketereh. After the propolis was crushed and ground into smaller pieces, the propolis was soaked in methanol for three days at a sample-to-solvent ratio of 1:10. The resulting solution was then filtered through a Whatman filter paper No. 1 and concentrated by using a rotary evaporator, under reduced pressure at 40 °C and named methanolic extract (ME). The methanolic extract then undergoes fractionation with three different solvents: hexane (HEX: FR), dichloromethane (DCM: FR) and methanol (MeOH: FR) and is kept cool in a freezer at -20 °C for further analysis. The physical properties of the extracts and fractions were observed. The percentages of extraction yield obtained were calculated using the following formula and recorded in Table 2 [19]:

% Yield of samples = [Weight of extracts (g) / (Weight of raw propolis (g)] x 100%

2.4 Determination of Total Phenolic Content (TPC)

Folin estimated the TPC in propolis extracts and their fractions using the Ciocalteu colourimetric method. Approximately, A volume of 60 µL of propolis extract was taken in test tubes for each sample. A volume of 200 µL of Folin–Ciocalteu reagent was added to the solution and allowed to incubate for 5 min at room temperature. After that, 800 µL of 7.5% Na_2CO_3 solution was added, thoroughly mixed and incubated protected from light for 2 h at room temperature. Gallic acid was used as a standard with concentrations that ranged from 7.81 to 500 µg/mL. Finally, an Enzyme-linked Immunosorbent Assay (ELISA) microplate reader was used to measure the absorbance at 765 nm. The TPC value was calculated by using the gallic acid standard curve, and the TPC values were calculated and expressed in milligrams of gallic acid equivalent (GAE) per mg of the extract (mg GAE/g) [19].

2.5 Determination of Total Flavonoid Content (TFC)

The TFC was estimated utilising the colourimetric method. Briefly, 140 µL of the extracts were taken and put into test tubes for each sample. A volume of 150 µL of 10% $AlCl_3 \cdot 6H_2O$ solution was added. 150 µL of 1M potassium acetate (CH_3CO_2K) and 260 µL of methanol were added to each sample. Finally, the mixture was vortexed and incubated for 30 min, and an ELISA microplate reader was used to measure the value of absorbance at 415 nm. Quercetin was used as a standard with concentrations that ranged from 7.81 to 500 µg/mL. The TFC value was calculated using the quercetin standard curve, and the TFC values were calculated and expressed in milligrams of quercetin equivalent (QE) per gram of extract (mg QE/g) [19].

2.6 Antioxidant Activity

2.6.1 DPPH Free Radical Scavenging Activity

A 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used to assess the extracts' and their fractions' antioxidant activity. This reagent functions as a detector for antioxidants by turning purple into yellow or colourless depending on the hydrogen donor or electron transfer of the compound. The antioxidant activity of the methanolic extracts and their fractions was assessed and determined using the DPPH radical scavenging test method, with slight adjustments [19]. In a nutshell, 0.125 mM of DPPH dissolved in methanol was generated as the stock solution. The samples and the DPPH stock solution were allowed to incubate and protected from light for 30 min at room temperature. An ELISA microplate reader was used to measure the value of absorbance at 517 nm using DMSO as a blank. All experiments were run in triplicate. Trolox and quercetin were also tested under the same conditions. The following mathematical equation was used to determine the percentage of DPPH scavenging activity:

$$\% \text{ DPPH scavenging activity} = [1 - (X_2/X_1)] \times 100$$

Where X_1 is the value of absorbance for the blank, and X_2 is the value of absorbance for the propolis sample after reaction.

2.6.2 ABTS Free Radical Scavenging Activity

The ABTS cation radical scavenging method, as reported by Maroof *et al*. [20], with alteration, was used to determine the percentage of ABTS radical scavenging activity. By reacting 2.45 mM of potassium persulfate dissolved in deionised water with a 7 mM ABTS aqueous solution, the ABTS cation radical was created. Before use, the mixture could stand for 12 to 16 hours at room temperature, away from light. The ABTS cation radical solution was diluted with methanol on the day of analysis, resulting in an absorbance value of 0.70 (± 0.02) at 734 nm. The mixture of samples and ABTS radical solution was left to incubate for 6 min, protected from light, and an ELISA microplate reader was used to measure the value of absorbance at 734 nm using DMSO as a blank. All experiments were run in triplicate. Trolox and quercetin were also tested under the same conditions as standards. The following mathematical equation was used to determine the percentage of ABTS scavenging activity:

$$\% \text{ ABTS scavenging activity} = [1 - (X_2/X_1)] \times 100$$

Where X_1 is the value of absorbance for the blank, and X_2 is the value of absorbance for the sample after reaction.

2.6.3 Ferric Reducing Antioxidant Power Activity

The antioxidant activity of the propolis samples was assessed by using the ferric reducing antioxidant power (FRAP) assay. The procedure is based on calculating the propolis's ability to reduce iron [21]. A 0.3 M acetate buffer, pH 3.6 (achieved by adding glacial acetic acid, sodium acetate trihydrate and distilled water) added with 0.01 mM TPTZ (0.031 g of TPTZ in 0.04 M HCl) and 0.02 M $FeCl_3$ ($FeCl_3.6H_2O$ dissolved with distilled water). Acetate buffer, $FeCl_3$ solution and TPTZ solution were combined in a ratio of 10:1:1 to create the working FRAP solution, which was then warmed up in a water bath to 37 °C. The mixture of samples and FRAP working solution was incubated for 15 min in the dark. Then, the value of absorbance using DMSO as a blank was measured at 593 nm by using an ELISA microplate reader. An aqueous solution of $FeSO_4$ ($FeSO_4.7H_2O$ dissolved with distilled water) was used for the calibration curve, with concentration ranging from 0.2 to 1.6 mM, while

quercetin and gallic acid were also tested under the same conditions as standards. All experiments were run in triplicate.

2.7 Nitric Oxide Radical Scavenging Activity

The Nitric oxide (NO) radical scavenging activity starts with the reaction mixture containing 80 µL of 0.01 M sodium nitroprusside (SNP) with phosphate buffer (pH 7.4) that was mixed with 40 µL of sample solution and incubated under direct light for 150 min at 25 °C. After the incubation period was over, 50 µL of 0.33% sulphanilic acid reagent (sulphanilic acid dissolved in 20% glacial acetic acid) was added to it and kept for 5 min protected from light. Then, 50 µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) was added and incubated for 10 min at 25 °C protected from light [22]. An ELISA microplate reader was used within 30 minutes after reaction to measure the absorbance at 540 nm using DMSO as a blank. All experiments were run in triplicate. Quercetin, as the standard, was also tested under the same conditions. The following mathematical equation was used to determine the percentage of NO scavenging activity:

$$\% \text{ NO scavenging} = [(1 - (X_2/X_1))] \times 100$$

Where X_1 is the value of absorbance for the blank, and X_2 is the value of absorbance for the sample after reaction [22].

2.8 Statistical Analysis

All experiments were performed thrice, and the results were averaged. Data were expressed as mean ± standard deviation (SD). The IC_{50} was calculated by using XY analyses: fit spline/LOWESS using Prism 8.

3.0 RESULTS AND DISCUSSION

The propolis activity may be affected by the extraction techniques. Solid-liquid extraction using methanol, ethanol, or water is the most widely used technique and influences the composition, yield percentage, and biological activities. Based on Figure 1, the colour of the samples was observed and recorded in Table 2. All samples were gummy and sticky, except for MeOH: FR (A), with solid and sticky. Based on Table 2, the methanolic extracts for both Besut Apiary (A) and Ketereh (K) have the highest yield with 33.70% and 34.29%, respectively, due to methanol, a polar solvent that dissolves all the content in the propolis. Research done by Pujirahayu *et al*. [23], showed that the solvent polarity can affect the yield of the propolis extracted. 70% ethanol yields the highest amount of 18.33 ± 1.82% compared to propylene glycol (15.88 ± 0.48%), water (15.33 ± 0.54%), virgin coconut oil (14.22 ± 0.22%), and olive oil (14.06 ± 1.07%). Zin *et al*. [19], showed that a comparison using maceration extraction for three days with 70% ethanol and 95% ethanol yielded a significant outcome in terms of yield, as 70% ethanol achieved only 7.05%, while 95% ethanol achieved 36.48%. Mokhtar *et al*. [24], showed the comparison of three different sample-to-solvent ratios with water and ethanol. For ethanol, 1:10 was the best outcome with 10.67% compared to 1:5 (5.79%) and 1:15 (4.20%). The same trend was also displayed when using water, as 1:10 had the highest yield of 9.05% compared to 1:5 (5.95%) and 1:15 (7.16%). Methanol fractions (MeOH: FR (A) & MeOH: FR (k)) had the lowest yield for the fractions due to the majority of the content from the methanolic extract being dissolved by both hexane and DCM, leaving the polar compounds within the methanol fraction.

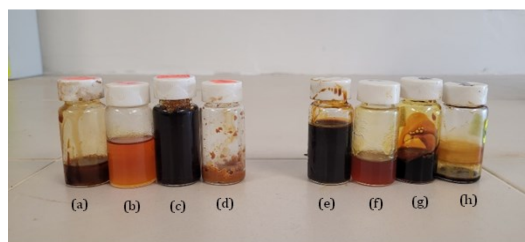

Figure 1 The extracts and fractions of *G. thoracica* propolis; (a)ME: A, (b) HEX: FR (A), (c) DCM: FR (A), (d) MeOH: FR (A), (e) ME: K, (f) HEX: FR (K), (g) DCM: FR (K) and (h) MeOH: FR (K)

Table 2 The value of percentage yield of the extracts and their fractions for both Besut Apiary (A) and Ketereh (K) propolis

Sample	Besut Apiary (A)		Ketereh (K)	
	Colour	% yield	Colour	% yield
Methanolic extract	Brown	33.7	Dark Brown	34.29
Hexane fraction	Yellowish Brown	9.13	Light Brown	8.47
Dichloromethane fraction	Dark Brown	20.1	Dark Brown	22.86
Methanol fraction	Light Brown	1.32	Dark Brown	1.05

3.1 Total Phenolic Content (TPC)

The quantities of TFC and TPC of the methanol-extracted and the fractions of *G. thoracica* propolis were analysed and determined in the current study, since propolis extracts are abundant with polyphenols. The quantities of TPC and TFC of the methanol-extracted and the fractions of *G. thoracica* propolis were analysed and determined in the current research. Badiazaman *et al*. [7], found that the phenolic and flavonoid content directly correlates with antioxidants and antiradical activity, with propolis from Besut (BST) having the highest flavonoid content and DPPH scavenging activity

compared to Gua Musang (GM), Tanah Merah (TM), Lundang (LDG) and Dungun (DGN). The presence of phenolic compounds, especially flavonoids, the most frequent and effective antioxidant chemicals, seemed to correlate with DPPH and ABTS radical scavenging activity.

Galangin was less active due to the structure of the compound with only three hydroxyl groups, whereas the most active flavonoids, such as kaempferol and quercetin, had four or five hydroxyl groups that added to their antioxidant qualities [7]. Galangin is one of the flavonoid constituents of propolis which is also associated with antibacterial and antiviral activity. Governa *et al*., (2019) study focuses on the ethanolic extract of propolis, which contains a compound named galangin that can against a subtype of the Hemagglutinin Type 1 and Neuraminidase Type 1 (H1N1) virus *in vitro*. The outcome of the study was the extract (35 µg/mL) restrict the activity of enzyme called neuraminidase (IC_{50}, 35.29 µg/mL) [25], and quercetin was found to be active against rhinovirus-2 while kaempferol reduced the viral Ribonucleic acid (RNA) reproduction of rhinovirus-3 virus in Henrietta Lacks (HeLa) cells and inhibited the cells being penetrated by the viruses [26]. Table 3 shows the total phenolic contents of extracts and their fractions obtained from two localities: Besut Apiary (A) and Ketereh (K). A calibration curve was plotted to determine and calculate the TPC value of the methanolic extracts and their fractions, with a regression equation of $y = 0.0061x + 0.391$ and $r^2 = 0.9949$.

Table 3 TPC value of propolis methanolic extracts and their fractions

Propolis Samples	TPC value (mg GAE/g)
ME (A)	69.40 ± 0.06
HEX: FR (A)	10.33 ± 0.01
DCM: FR (A)	41.45 ± 0.01
MeOH: FR (A)	179.13 ± 0.03
ME (K)	104.32 ± 0.05
HEX: FR (K)	11.48 ± 0.08
DCM: FR (K)	19.89 ± 0.02
MeOH: FR (K)	99.84 ± 0.04

In Table 3, ME (K) with 104.32 ± 0.05 mg GAE/g had the higher TPC value compared to ME (A), which had the lower phenolic content at 69.4 ± 0.06 mg GAE/g. The TPC value for fractions, both methanol fractions (MeOH: FR (A) and MeOH: FR (K)), had a higher value than other fractions. Badiazaman *et al*. [7] demonstrated a lower TPC value of 9.23 ± 0.37 mg/mL GAE. However, Idris *et al*. [21], showed three different TPC values of 302.21 ± 0.11 mg/mL GAE, 156.79± 0.06 mg/mL GAE and 111.38 ± 0.06 mg/mL GAE, which indicate the obtained results were consistent. Studies have demonstrated that propolis phenolic components can effectively increase biological effects like antibacterial, anti-inflammatory, and anticancer properties while improving human health [7].

3.2 Total Flavonoid Content (TFC)

A quercetin standard curve calibration was obtained from the absorbance of different concentrations of quercetin (7.81 to 500 µg/mL). The TFC values of methanolic extracts and their fractions expressed in quercetin equivalent (mg QE/g) were determined and calculated from the graph of absorbance against quercetin concentration plotted, $y = 0.0051x + 0.0049$ and $r^2 = 0.9962$. In Table 4, the highest TFC value from methanolic extracts was ME (A) with 159.1 ± 0.03 mg QE/g, while ME (K) had a lower TFC value of 119.5 ± 0.01 mg QE/g. For the fractions, both methanol fractions had the highest values of TFC among the fractions, with 375.05 ± 0.05 mg QE/g for MeOH: FR (A) and 193.42 ± 0.06 mg QE/g for MeOH: FR (K). Idris *et al*. [21], demonstrated three different TFC values of 99.08 ± 0.03 mg/mL QE, 73.08 ± 0.01 mg/mL QE and 64.68 ± 0.02 mg/mL QE. The TFC results in this study, however, are slightly less than the results of Adli *et al*. [27], who found a TFC value of 435.00 ± 6.57 mg/mL QE. This indicates the obtained results are consistent. The differences in both TPC and TFC values for the samples are due to factors such as the originality of raw material and variations in the stingless bees preferred local flora [7].

Table 4 TFC value of propolis methanolic extracts and their fractions

Propolis Samples	TFC value (mg GAE/g)
ME (A)	159.10 ± 0.03
HEX: FR (A)	86.56 ± 0.05
DCM: FR (A)	121.52 ± 0.04
MeOH: FR (A)	375.05 ± 0.05
ME (K)	119.50 ± 0.01
HEX: FR (K)	91.39 ± 0.07
DCM: FR (K)	125.05 ± 0.03
MeOH: FR (K)	193.42 ± 0.06

3.3 Antioxidant Activity

3.3.1 DPPH Free Radical Scavenging Activity

This ability to "catch free radicals," or radical-scavenging activity, is evaluated by the DPPH assay. Biological targets of oxidative stress should be protected by compounds with strong radical scavenging activity, which can deactivate free radicals in a variety of ways (e.g., by producing a less reactive form of radical) and usually operate as electron donors [28]. An equation that relates the concentration of the methanolic extracts and their fractions to the proportion of trapped radicals was used to get the IC_{50} values. A lower value of IC_{50} indicates a stronger DPPH radical inhibition and, thus, higher antioxidant activity. In our research in Figure 2, the highest percentage of DPPH radical scavenging

activity was observed for propolis samples from Besut Apiary for both the methanolic extract and fractions. In Figure 2, both quercetin and trolox have the inhibition of 84.97 ± 0.01% with IC_{50} of 7.51 µg/mL and 86.36 ± 0.01% with IC_{50} of 6.63 µg/mL, respectively. For the methanolic extract, ME (A) was observed to reach the highest inhibition at 61.54 ± 0.04% with an IC_{50} value of 203.72 µg/mL, while ME (K) was at 50.93 ± 0.01% with an IC_{50} value of 477.44 µg/mL. For the fractions, the methanol fraction (MeOH: FR (A) and MeOH: FR (K)) had the highest DPPH free radical scavenging activity compared to the HEX (HEX: FR (A) and HEX: FR (K)) and DCM (DCM: FR (A) and DCM: FR (K)) fractions. Both HEX fractions are considered inactive, while DCM: FR (A) is the only one with 65.68 ± 0.01% and an IC_{50} value of 185.84 µg/mL. Both methanol fractions are active, especially MeOH: FR (A) with 70.89 ± 0.01% and an IC_{50} value of 118.11 µg/mL, while MeOH: FR (K) has a lower inhibition of 56.46 ± 0.01% and an IC_{50} of 336.26 µg/mL. This indicates that the higher the TFC value, the higher the radical scavenging and antioxidant activities. Research conducted by Badiazaman *et al*. [7], showed that BST propolis with the highest TFC value of 17.22 ± 0.16 mg QE/g extract outperformed GM propolis for DPPH scavenging activity, which had the highest TPC value of 23.43 ± 0.50 mg GAE/g extract.

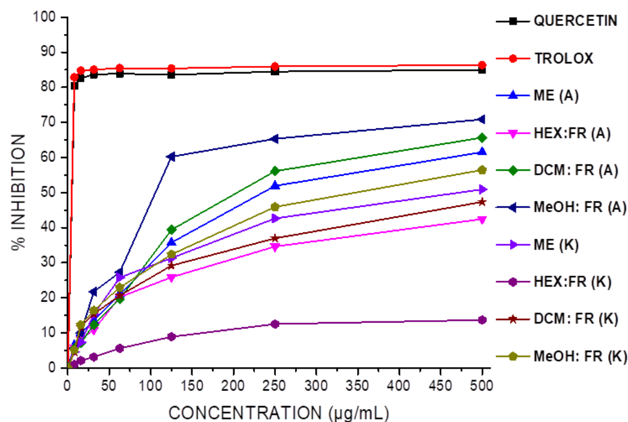

Figure 2 Graph plot for percentage of inhibition against concentration (µg/mL) showing DPPH scavenging activity for methanolic extracts and their fractions

3.3.2 ABTS Free Radical Scavenging Activity

In general, the idea behind an ABTS scavenging assay is comparable to that of the DPPH scavenging assay, which also makes use of free radical affinity activity. The oxidation of potassium persulfate with ABTS salt produces the ABTS scavenging test, which is reduced in the presence of an antioxidant that donates hydrogen [29]. The equation used to determine the IC_{50} values links the percentage of trapped radicals to the concentration of the methanolic extracts and their fractions. Stronger suppression of the DPPH radical and hence higher antioxidant activity is indicated by a lower IC_{50} value. The Besut Apiary samples in our study had the maximum radical ABTS scavenging activity for both the methanolic extract and the fractions shown in Figure 3. Both quercetin and trolox have the inhibition of 93.1 ± 0.01% with IC_{50} of 4.7 µg/mL and 96.84 ± 0.01% with IC_{50} of 4.56 µg/mL, respectively. ME (A) was slightly higher than ME (K) for the methanolic extract, with 96.7 ± 0.01% with IC_{50} values of 28.24 µg/mL and 96.29 ± 0.01% with IC_{50} values of 34.76 µg/mL, respectively. For the fractions, both DCM: FR (A) and MeOH: FR (A), which came from Besut Apiary, had the highest inhibition of 96.65 ± 0.01% with IC_{50} values of 27.78 µg/mL and 96.73 ± 0.01% with IC_{50} values of 27.68 µg/mL, respectively. Both methanol fractions (MeOH: FR (A) and MeOH: FR (k)) have a higher percentage of inhibition compared to quercetin and slightly lower compared to trolox, but both have higher IC_{50} values compared to quercetin and trolox.

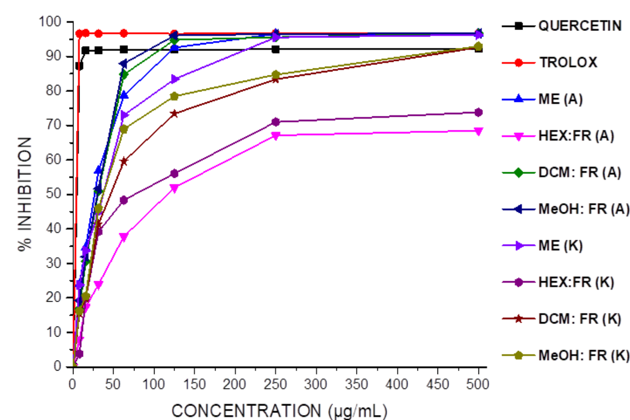

Figure 3 Graph plot for percentage of inhibition against concentration (µg/mL) showing ABTS scavenging activity for methanolic extracts and their fractions

3.3.3 Ferric Reducing Antioxidant Power Activity

Potential antioxidant activity was evaluated by using ferric reducing antioxidant power (FRAP) assay, which is based on the capability of polyphenol compounds to change ferricyanide $[Fe(CN)_6]^{3-}$ into ferrocyanide $[Fe(CN)_6]^{4-}$. This research is predicated on the ability of antioxidants to produce a blue complex by converting ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). A significant antioxidant activity or reductive power is indicated by a high absorption at 700 nm. The antioxidant activity of tea extracts and red wines, Fe^{2+} equivalents, has been measured using the FRAP value. Nevertheless, contrary to what some authors have claimed, the reducing ability does not always directly correspond to the antioxidant activity [30]. The FRAP values of methanolic extracts and their fractions expressed in $FeSO_4$ equivalent in µM (Fe^{2+}/extract) were determined from the calibration curve of absorbance against $FeSO_4$ concentration plotted, $y = 0.0002x + 0.0859$ and $r^2 = 0.9967$. Both

quercetin and gallic acid have a reducing ability of 310.33 ± 3.1 in µM (Fe^{2+}/extract) and 351.54 ± 3.52 in µM (Fe^{2+}/extract), respectively. For methanolic extracts in Figure 4, ME (K) had double reducing activity compared to ME (A) with 20.21 ± 0.2 in µM (Fe^{2+}/extract) and 10.71 ± 0.11 in µM (Fe^{2+}/extract), respectively. This indicates that a higher TPC value, higher reducing power. Asem *et al*. [29], demonstrated that *G. thoracica* with a TPC value of 55.16 ± 7.52 µM Gallic acid/g dry weight had higher reducing ability compared to *H. itama* (34.17 ± 1.52 µM Gallic acid/g dry weight) and *T. apicalis* (28.57 ± 3.17 µM Gallic acid/g dry weight). For fractions, both methanol fractions had the highest reducing activity, with 32.78 ± 0.33 µM (Fe^{2+}/extract) from MeOH: FR (A) and 16.74 ± 0.17 µM (Fe^{2+}/extract) from MeOH: FR (K). The current propolis extracts and fractions in the FRAP assay showed a weak absorbance but were consistent. Asem *et al*. [29], showed a good absorbance with *G. thoracica* propolis with 587.044 µM Trolox/g dry weight compared to *T. apicalis* (587.044 µM Trolox/g dry weight) and *H. itama* (405.617 µM Trolox/g dry weight). Mohtar *et al*. [30], conducted a comparison of propolis from Venezuela, Argentina, and Brazil, showing FRAP values as low as 11.8 ± 0.6 µmol AA/g and values as high as 295.3 ± 0.7 µmol AA/g. This suggests that the stingless bee species and the vegetation at the collection site may have an impact on the antioxidant and reducing capacity.

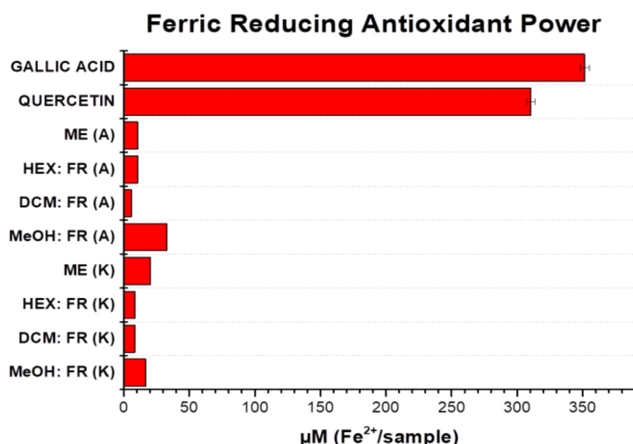

Figure 4 FRAP activity of methanolic extracts and their fractions

3.4 Nitric Oxide Radical Scavenging Activity

Quercetin had an inhibition of 79.54 ± 0.01%. For the methanolic extracts in Figure 5, ME (A) had a higher inhibition at 62.5 µg/mL compared to ME (K) with 30.99 ± 0.05% and 23.76 ± 0.04%, respectively. MeOH: FR (A) had the highest inhibition for fractions at 250 µg/mL with 37.94 ± 0.01%. NO can also be influenced by certain types of compounds, such as phenolic, flavonoids and labdane-type diterpenes, which are effective in nitric oxide inhibition [31]. That may not be the case for propolis produced by both localities, as none of the extracts and fractions reach 50% of scavenging activity.

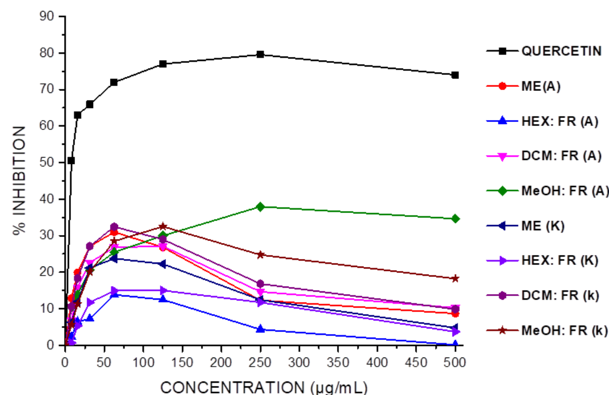

Figure 5 Graph plot for percentage of inhibition against concentration (µg/mL) showing NO scavenging activity for methanolic extracts and their fractions

Propolis from Besut Apiary had the highest radical scavenging activity, antioxidant activities and NO scavenging activity. These variations might be due to the differences in botanical sources, which affect the chemical composition of the methanolic extracts and their fractions. A study by Badiazaman *et al*. [7], compared 5 localities by using *G. thoracica*'s propolis, showing that BST propolis had the highest DPPH radical scavenging compared to other localities. A study conducted by Abdullah *et al*. [1], comparing *G. thoracica*, *H. itama,* and *T. binghami* found that *H. itama* had a lower IC_{50} value for DPPH radical scavenging activity due to its high phenolic content. This indicates that phenolic compounds, regardless of the synergetic effects among the terpenes, aromatic acids and flavonoids, these compounds play a major role in the antioxidant activity. Research conducted by Idris *et al*. [21], found that free radical scavenging activity, such as DPPH and ABTS is directly influenced by flavonoid and phenolic content, indicating the correspondence between TFC and TPC value with the antioxidant activity of the propolis extracts. The main cause of this is the presence of aromatic hydroxyl groups in the propolis, which have potent electron-accepting properties. The isolation process by Badiazaman *et al*. [32], via column chromatography (CC), was conducted for the first time by using the propolis of *G. thoracica* led to five compounds: cycloartenol, mangiferolic acid, mangiferonic acid, ambolic acid and ambonic acid. The samples undergo cytotoxic activity against HepG2 and MCF-7 cancer cell lines, and mangiferolic acid showed strong cytotoxicity for both cell lines with IC_{50} of 4.82 and 5.08 µg/mL, respectively. Thus, mangiferolic acid represents the most active compound in the extract of *G. thoracica* and could serve as a bioactive marker as well.

4.0 CONCLUSION

This study presented the results of antioxidants (DPPH, ABTS, FRAP), NO scavenging activity and the determination of TPC and TFC value of *G. thoracica* propolis collected from two localities: Besut Apiary (A) and Ketereh (K). As a result, the methanol fraction from Besut Apiary (MeOH: FR (A)), the highest flavonoid and phenolic content, was the best in antioxidants and NO scavenging activity, indicating a good source of antioxidants and some anti-inflammatory properties. Propolis standardization requires more research on phytochemical screening and the identification of propolis components with biological activity.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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