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IN-VITRO EVALUATION NITRIC OXIDE INHIBITORY ACTIVITY AND PHYTOCHEMICAL SCREENING OF *Heterotrigona itama* PROPOLIS

NURUL AMIRAH ROSMAN[1,2], NOR ATIQAH JUSRIL[1]*, MUHAMMAD AMIR FIKRI MD FAUZI[1] AND KHAMSAH SURYATI MOHD[1]*

[1]School of Agriculture Sciences and Biotechnology, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, Malaysia. [2]Faculty of Dentistry, Universiti Sains Islam Malaysia, Pandan Indah Campus, Level 18, Tower B, Persiaran MPAJ, Jalan Pandan Indah Utama, 55100 Kuala Lumpur, Malaysia.

**Corresponding authors: atiqahjusril@unisza.edu.my, khamsahsuryati@unisza.edu.my*
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Abstract: The therapeutic benefits of propolis have been known and utilised since ancient times. *Heterotrigona itama* is the most abundant stingless bee in Peninsular Malaysia and in this study, its propolis was subjected to successive maceration extraction methods with different solvents. The chemical composition of propolis solvent fractions was then evaluated using phytochemical screening analysis. After that, the fractions were assessed for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) before being subjected to Nitric Oxide (NO) assay to determine their anti-inflammatory potential. The most active fraction in the NO assay was then selected to determine its cytotoxicity on RAW 264.7 macrophage cells. In phytochemical screening analysis, thin-layer chromatography showed the presence of flavonoids in the Methanol Fraction (MFR). The TPC and TFC results showed that MFR had the highest content of phenolics and flavonoids, which was 54.093 ± 0.003 µg/mL and 30.15 ± 0.003 µg/mL, respectively. The MFR was also non-toxic and could increase the proliferation of RAW 264.7 cells. The MFR possessed moderate anti-inflammatory properties as demonstrated with IC_{50} values of 82.87 µg/mL and 92.31 µg/ml in NO-inhibitory activity and cell-based assays, respectively, compared to other fractions.

Keywords: *Heterotrigona itama*, stingless bee propolis, phytochemicals, anti-inflammatory.

Introduction

Stingless bees are eusocial bees found in tropical and subtropical regions that belong to the Meliponini tribe (Abdullah *et al*., 2019). Propolis is one of the most valuable products of the bee, with many potential benefits that have yet to be discovered. Propolis is a nutrient rich colloidal substance produced by the bees' glandular fluids after reacting with the nectar of plant buds and sand. It is believed that the bees produce propolis to maintain, safeguard and prolong the health of their colonies (Zhang *et al*., 2020). It comprises half resins and vegetable balms, half beeswax, 5% pollen, and 10% essential and aromatic oils (Anjum *et al*., 2019). When cold, propolis is a hard and brittle resin. When warm, it becomes soft, flabby, and extremely sticky; it also comes in a variety of colours, including brown, green, and red (Marcucci, 1995; Rivera-Yañez *et al*., 2020).

Propolis may consist of more than 150 compounds, which include polyphenols, flavonoids, aglycones, phenolics, and ketones (Rozman *et al*., 2022). *H. itama* is a common stingless bee species in Malaysia that serves as an essential crop pollinator (Mohd & Zin, 2020). It's propolis has been found to contain aromatic acids, alcohols and terpenes (Kasote *et al*., 2019). Phenolic acid and flavonoids are two well-known bioactive chemicals in propolis, both are known anti-oxidants that are being studied for their antimicrobial, anti-inflammatory, and anticancer activities in cells (Mendonça *et al*., 2020; Syahariza *et al*., 2022). Furthermore, propolis contains more flavonoids and phenolic acids than honey, implying that it is a potent anti-oxidant and anti-inflammatory substance (Afonso *et al*., 2020).

Inflammation is a complex immunological mechanism that manifests as an overreaction to a benign stimulus (Al-Hatamleh *et al*., 2020). It is an important aspect of the host immune response that protects the body against a variety of harmful insults such as pathogen infection and the toxicity of their metabolism products, besides being part of the healing process in damaged cells and tissues (Lu *et al*., 2020). As for propolis, it possesses medicinal properties that have been used since antiquity (Brodkiewicz *et al*., 2020).

Nowadays, propolis is a natural remedy sold in many health food stores mainly for topical use. It is also used in cosmetics as well as traditional medicine (Wagh, 2013). It has been observed to have inflammation-modulating effects (Zulhendri *et al*., 2022) research has demonstrated the efficacy propolis as a potential raw material for pharmaceuticals and nutraceuticals. There is limited report detailing the mechanisms of action of propolis and its bioactive compounds in relation to their anti-inflammatory properties. Thus, the aim of the present review is to examine the latest experimental evidence (2017-2022) such as suppressing the expression of inflammatory genes like matrix metalloproteinase-7 (*Mmp7*), Epidermal Growth Factor Receptor (*EGFR*), and Adrenomedullin (*Adm*) while increasing the expression of other inflammatory genes like Caveolin-1 (*Cav1*), Calmodulin-1 (*Calm1*), and Tumour Necrosis Factor (*TNF*) (Bueno-Silva *et al*., 2017).

Although studies on the medicinal properties of propolis have been conducted, few can relate to its anti-inflammatory activities, particularly for products produced by *H. itama*. In this study, methanol, hexane, and dichloromethane were used to separate the propolis into different polarities. Phytochemical assays were also conducted to identify the group of compounds that are present in each fraction of propolis. The identification of a class of compounds will further strengthen the correlation between the group of phytochemicals and anti-inflammatory activities. The RAW264.7 macrophage cell line was used to assess the inhibition of anti-inflammatory genes and toxicity of propolis. The cells were then activated with lipopolysaccharides (LPS) due to the inflammatory effects it allegedly produces by activating Toll-Like Receptor 4 (TLR4) signalling (Tucureanu *et al*., 2017). The findings of the present study may indicate that *H. itama* propolis has the potential to be a powerful anti-inflammatory agent, particularly for wound healing.

Materials and Methods

Chemicals

Solvents and reagents like ethanol (95%, analytical grade), methanol (100%, analytical grade), ethyl acetate (analytical grade), dimethyl sulfoxide (DMSO), sodium nitroprusside (SNP), and n-hexane (analytical grade) were purchased from Merck (Merck KGaA, Darmstadt, Germany). Quercetin, vanillin, and gallic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) were purchased from Nacalai Tesque (Nacalai Tesque Inc., Kyoto, Japan). Fetal Bovine Serum (FBS) and penicillin-streptomycin were obtained from Gibco (Thermo Fisher Scientific, MA, USA).

Propolis Collection and Preparation

Approximately 600 g propolis from *H. itama* stingless bees was used in the experiment. The propolis was collected from the apiary of Universiti Sultan Zainal Abidin (UniSZA) Besut campus in the state of Terengganu, Malaysia. The sample was stored at 4°C.

The propolis was crushed using a pestle and mortar. Then, the crushed sample was soaked in 100% methanol in a conical flask for three days and stirred with a spatula daily. After that, the solution was filtered and the propolis powder was soaked again in methanol until the solution turned light yellow. The second solution was also filtered and the filtrates were combined and placed on a rotary evaporator to obtain the methanolic crude extract.

The fractionation of propolis methanolic crude extract was conducted using three solvents: 100% methanol, hexane, and dichloromethane (Badiazaman *et al*., 2019). Each fraction was weighed and kept at 4°C. The fractions a were bottled and labelled as methanol crude (MCR), hexane crude (HFR), dichloromethane (DCM) fraction, and methanol fraction (MFR). The samples were then stored at 4◦C. The percentage of yield was calculated in Equation 1 (Mokhtar, 2019):

$$\text{Percentages of yield (\%)} = \frac{\textit{Weight of dried sample } (g)}{\textit{Weight of propolis } (g)} \text{ x } 100 \qquad \text{(Equation 1)}$$

Phytochemical Screening Using Thin Layer Chromatography

Different fractions of *H. itama* propolis were screened to identify the compounds in each fraction according to the standard screening method. A total of 30 mg of each fraction was dissolved in 1 ml of the respective solvents. Then, samples were sonicated for 30 minutes and centrifuged (10 minutes, 140 rpm). Two type of plates were used, which were labelled as developed and derivated plates. The developed plate was prepared in a glass twin-through chamber and filled with toluene, ethyl acetate, acetic acid, and methanol in a ratio of 6:4:0.2:0.4 (v/v/v/v). After that, the plates were dried using a heat gun and observed under 254 nm and 366 nm. After observation, the plates were sprayed with different chemical reagents, including vanillin-sulphuric acid, p-anisaldehyde, and iodine to identify specific chemicals (Badiazaman *et al*., 2019).

Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was quantified as milligrams of gallic acid equivalents per gram of sample extract (mg GAE/g extract). A 1 mg/mL gallic acid stock solution was prepared and serially diluted to produce final concentrations of 60, 80, 100, 120, 140, 160, 180, and 200 mg/mL. A stock solution of 5 mg/mL was prepared for propolis samples and 60 L of the stock solution was pipetted into samples to produce a final concentration (1 mg/mL) of sample extract. A total of 200 µL of Folin-Ciocalteu reagent was added to the samples before vortexing. Then, 800 µL of 7.5% sodium carbonate (Na_2CO_3) was added and thoroughly mixed until the solution turned blue. The samples were left at room temperature (37°C) in the dark for two hours. Finally, the absorbance of the mixture was measured using a spectrophotometer with a 765 nm wavelength (Zin *et al*., 2018).

Total Flavonoid Content (TFC)

The Flavonoid Concentration (TFC) was quantified as mg of quercetin equivalent (Q) per gram of sample extract. A standard accurately weighed 0.5 g/mL was dissolved in 1 mL of methanol (Zin *et al*., 2018). A total of 140 µL of the stock solution (5 mg/mL) of each propolis extract was added to achieve a final concentration of 1 mg/mL. Then, 150 µL of 10% aluminium chloride ($AlCl_3$) was added to the propolis extracts, followed by 150 µL of 1M potassium acetate, and topped up with distilled water to a final volume of 700 µL. The extracts were left to incubate in the dark for 30 minutes at room temperature. Quercetin was used as a positive control. Once the reaction was complete, the samples' absorbance was measured using a spectrophotometer at 415 nm (Zin *et al*., 2018).

Nitrite Oxide Assay (NO)

Each sample was diluted in 20 µL of dimethyl sulfoxide (DMSO) in a 96-well plate from well B to H, followed by 40 µL sample, and standard in well A. Then, well A was serially diluted to well E. After the serial dilution, 50 µL of sodium nitroprusside (SNP) was added, and incubated for one hour and 30 minutes under direct light. A total of 50 µL salicylic acid was added after one hour 30 minutes and incubated for another

five minutes. Lastly, 50 µL of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) was added and the samples were incubated for 10 minutes in the dark. Quercetin was used as a standard. The absorbance was measured using a spectrophotometer at 540 nm and the extract's percentage of NO radical inhibition was calculated.

Cell Viability Assay

Cell viability was assessed in an MTT assay, which measured the amount of dehydrogenase activity that remained in cultured cells (Karem *et al*., 2021). RAW 264.7 cells were seeded in 96-well plates at 50 x 10^5 cells/well and cultured for 24 hours. After the confluence reached 85-90%, the cells were exposed to different levels of concentrations of propolis samples (7.812 -1,000 µg/ml) and incubated for another 24 hours. The cells without any treatment were used as the negative control. The cells were then treated with 5 mg/ml MTT for 3-4 hours under indirect light. DMSO was used to stop the reaction. The absorbance was measured at 570 nm using a microplate reader (Jusril *et al*., 2022).

Determination of Nitrite Production

RAW 264.7 macrophage cells were seeded at a density of 50 x 10^5 cells/well in a 96-well plate. Cells were then pre-treated with various concentrations of samples before being stimulated for 24 hours at 37°C. The concentration used was 15.62 µg/ml - 1,000 µg/ml for all samples. Pre-treated cells were induced with 4 µg/mL of LPS (Hafiz *et al*., 2020; Karem *et al*., 2021). The cell culture was then centrifuged and the supernatant was collected. The level of NO generation in the culture supernatant was determined using the Griess test. The test was performed by adding 50 µL of 1% sulphonamide, 50 µL of 0.1% NED, and 5% phosphoric acid to 100 µL of culture supernatant in each well of a 96-well microplate and incubated at room temperature for 15 minutes in the dark. Dexamethasone (DEXA) was used as a positive control. A standard curve was plotted using sodium nitrate and the NO concentration in the supernatant were calculated by extrapolating their absorbance to the standard curve which was measured using a spectrophotometer at 540 nm light wavelength.

Statistical Analysis

Assays were performed in triplicates and the results were expressed as mean with standard deviation. All of the graphs were produced using GraphPad Prism v.9.0. (Graph-Pad Software, Inc., CA, USA). Significant differences between mean samples were determined using a one-way analysis of variance (ANOVA), followed by post-hoc Tukey test. Differences between the mean of the different samples and the controlled group were set at P value.

Results and Discussion

Percentage Yield

The solvent extraction process used to extract the specially selected chemicals in propolis was founded on the idea of ensuring the polarity of the solvent used was comparable to the polarity of the target compounds, allowing the specific chemicals to be successfully dissolved in the solvent (Zhang *et al*., 2020). Each solvent targeted a different type of compound. Raw propolis may not be utilised directly in production without being extracted and treated further (Zhang *et al*., 2020). Maceration is a traditional extraction procedure in which samples are soaked in a closed container and incubated at room temperature (Azwanida, 2015).

As a result, we utilised three commonly used solvents, namely methanol, hexane, and dichloromethane to extract the phytochemicals in propolis produced by *H. itama* (Badiazaman *et al*., 2019). The extraction yield of different solvents extraction is shown in Table 1, the extraction yield with HFR (8.16%) was greater in general than that with other solvents, significantly higher than that with DFR (3.28%), MCR (1.28%), and MFR (2%) ($p^* < 0.05$) compared to other samples. These results

revealed that the highly polar solvents had a higher extraction efficiency (Truong *et al*., 2019). It had been documented that varying extraction conditions could produce varying results in terms of yield and chemical composition. As a result, yield was a significant response variable that should be assessed while examining various techniques for extracting natural products (De Carvalho *et al*., 2020).

Methanol is a highly effective polar solvent that could be used to extract polar chemicals from plant materials and other biological matrices, including alkaloids, flavonoids, and phenolics. It is a frequently-used solvent due to its ability to dissolve the biochemical contents of organic samples (Chaves *et al*., 2020). Hexane is frequently used in the food sector for oil extraction since it is perfect for compounds that are insoluble in polar solvents. As a moderately polar solvent, dichloromethane is frequently used to extract semi-polar substances like lipids, alkaloids, and essential oils. Many organic molecules that are less soluble in very polar or non-polar solvents can be dissolved by dichloromethane due to its intermediate polarity (Huang *et al*., 2016).

Therefore, hexane produces a higher yield than other solvents since the key component of propolis is wax, which is a resinous substance that is more soluble in non-polar solvents. The yield of each solvent is stated in Table 1.

Thin Layer Chromatography Analysis

For substance identification contained in each fraction, Thin Layer Chromatography (TLC) was performed (Badiazaman *et al*., 2019). Spray reagents for chemical compound identification included vanillin-sulphuric acid, iodine, and p-anisaldehyde. Vanillin-sulphuric acid is a general-purpose reagent for detecting amines, amino acids, higher alcohols, phenols, and essential oils. Meanwhile, iodine is utilised in the detection of many chemical compounds because it has a strong affinity for both unsaturated and aromatic molecules such as phenolic steroids, esters, alkaloids, and polycyclic compounds (Rozman *et al*., 2022). As for p-anisaldehyde, it detects almost the same compound as vanillin-sulphuric acid. However, these various fractions of *H. itama* had shown different compounds present in each of them as isted in Table 2.

The presence of different compounds in each fraction is varied due to the solvents' polarity (Riyadi *et al*., 2023). To extract lipophilic compounds, hexane is frequently used, followed by dichloromethane, and finally methanol to target highly polar molecules (Tzanova *et al*., 2020). Based on Table 2, MCR and MFR both had flavonoid, terpenoids, coumarins, and other aromatic compounds. The current findings were consistent with Ibrahim *et al*. (2016), who conducted a phytochemical screening of propolis produced by *H. itama* and *G. thoracica*. Terpenoids, flavonoids, essential oils, unsaturated, and aromatic chemicals were found in *G. thoracica* propolis. These chemical compounds also varied for propolis produced in different regions, seasons, and botanical sources. Differences in chemical composition were also caused by the location and timing of propolis collection (Rozman *et al*., 2022). It has been observed that MFR had the highest flavonoid content than other solvents. Flavonoid is known to play an important role in anti-inflammation (Lopes *et al*., 2019).

Table 1: The percentages of yield of each *H. itama* sample

Samples	Percentages of Yield (%)
MCR	1.28
HFR	8.16****
DFR	3.28
MFR	2.00

Significant differences between all samples, p****< 0.001.

Table 2: The results of phytochemical screening of all *H. itama* propolis fractions

Constituents	MCR	HFR	DFR	MFR	Colour	Chemical/Spray Reagent
Flavonoids	+	-	-	+	Blue fluorescence	Anisaldehyde
Terpenoids	+	+	+	+	Pink	Anisaldehyde
Saponins	-	-	-	-	Dark bluish	Vanillin-sulphuric acid
Steroids	-	+	+	-	Bluish-green	Anisaldehyde
Coumarins	+	+	+	+	Light blue	UV 366 nm
Essential oil	-	-	-	-	Red and brown	Vanillin-sulphuric acid
Aromatic compounds	+	+	+	+	Yellow-brown	Iodine

+: Present, -: absent.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Polyphenols and flavonoids are the most prevalent bioactive molecules, capable of scavenging free radicals, and chelating metal ions, which can contribute to anti-inflammatory action (Syahariza *et al*., 2022). TPC was determined through a colourimetric modified Folin-Ciocalteu method, with gallic acid as a positive control. Gallic acid is a well-characterised and widely available phenolic compound that is frequently used as a standard or control in the determination of TPC. This method's fundamental concept is the formation of complicated blue chemicals and their light absorbance at a wavelength of 765 nm. To convert heteropoly acid (phosphomolybdate-phosphotungstate) in the Folin-Ciocalteau reagent into a molybdenum-tungsten complex, phenol or phenolic-hydroxy groups must be oxidised (Martono *et al*., 2019).

Therefore, complex ions $MO6^+$ and W^+ in Folin-Ciocalteu will undergo reduction, causing the reaction to turn blue (Zin *et al*., 2018). Measuring total phenolics and flavonoid content serves multiple purposes, ranging from assessing antioxidant and anti-inflammatory capacity and nutritional quality, to guide plant breeding efforts and supporting research on the health benefits of specific foods (Zin *et al*., 2018). While propolis showed strong antioxidant properties, this does not directly prove its anti-inflammatory effects. To confirm these effects, future studies should measure the effects of propolis on specific inflammatory markers. TPC values were calculated using a regression line and the gallic acid standard curve of $y = 0.014x + 0.0709$ ($R^2 = 0.9527$) and demonstrated in gallic acid equivalent (mg/mL GAE) as shown in Appendix 1.

Based on Figure 1, MFR has the highest phenolic content (54.093 ± 0.003 mg/ml), followed by MCR (53.593 ± 0.005 mg/mL), DFR (51.736 ± 0.002 mg/mL), and the lowest was HFR (30.021 ± 0.01 mg/mL). The solvent and extraction technique used can have an impact on the chemical composition of propolis extract (Valverde *et al*., 2023).

A flavonoid is the most important component in the phenolic group, which also includes flavones, flavonols, flavanols, anthocyanins, isoflavones, and flavanones (Annisava *et al*., 2019). These components have been studied for their significant antioxidant, anti-diabetic, anti-inflammatory, anti-cancer, and cardioprotective properties (Xiao *et al*., 2011).

Findings from this study indicated that MFR possessed the highest flavonoid content (30.15 ± 0.003 mg/mL), followed by HFR (24.87 ± 0.057 mg/mL), MFR (13.88 ± 0.068 mg/mL), and the lowest was DFR (2.15 ± 0.003 mg/mL) as shown in Figure 1. MFR obtained the highest result since methanol can efficiently

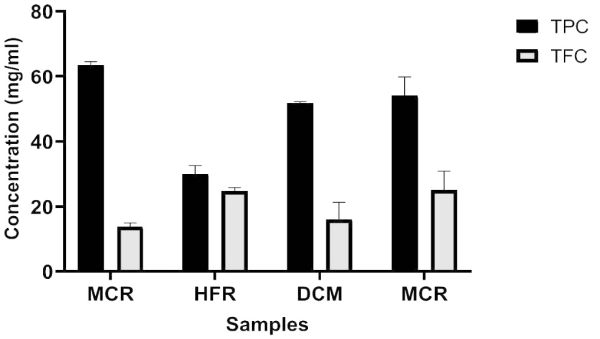

Figure 1: Comparison of TPC and TFC values of different solvent fractions of *H. itama* propolis

Table 3: The total phenolic content and total flavonoid content of each sample of propolis

Samples	Total Phenolic Content (TPC) mg/mL	Total Flavonoid Content (TFC) mg/mL
MCR	53.593 ± 0.005	13.88 ± 0.068
HFR	30.021 ± 0.01	24.87 ± 0.057
DFR	51.736 ± 0.002	2.15 ± 0.003
MFR	54.093 ± 0.003	30.15 ± 0.003

Note: Data shown in the table was gathered from three replicates and is presented as the mean standard deviation. TPC was measured in milligrams per gram of extract as gallic acid equivalent (GAE) mg/g while total flavonoid content was measured in milligrams per gram of extract as quercetin equivalent (QE)mg/g.

extract flavonoids, a polyphenolic component. Generally, organic solvents such as methanol, ethanol, acetonitrile, petroleum ether, acetone, water, and combinations of these solvents are used to extract flavonoids from plant matrices such as herbs, industrial residues, stems, and seeds (Dzah *et al*., 2020). Flavonoids have a wide range of benefits, especially in terms of anti-inflammatory activity (Yahfoufi *et al*., 2018).

TFC values were calculated using a regression line and the quercetin standard curve of $y = 0.0162x + 0.0773$, ($R^2 = 0.9993$) and demonstrated in quercetin equivalent (mg/mL GAE) as shown in Appendix 2. The anti-inflammatory activity of the samples can be attributed to the phenolic and flavonoid concentrations. Increased anti-inflammatory activity is caused by higher levels of phenolic and flavonoid content in the samples. The differences in TPC and TFC when using solvents like hexane, methanol, and dichloromethane (DCM) are mainly due to the solvents' polarities. More polar methanol extracts more phenolic and flavonoid compounds than less polar solvents like hexane and DCM. Higher extraction yields can lead to higher TPC and TFC, but the efficiency of the solvent in extracting specific compounds also plays a key role (Babbar *et al*., 2014).

Biochemical Nitric Oxide (NO) Assay

NO is an indicator to determine the antioxidant potential of peroxynitrite anion, which, when reacting with superoxide can break down to produce OH^- and NO^-. It's also been linked to neurotransmission, vesicular homeostasis, antimicrobial activity, and anticancer activity. (Zhang *et al*., 2020). In addition, NO is a signalling molecule that plays a role in many physiological processes, particularly inflammation (Zhang *et al*., 2020). The sodium nitroprusside spontaneously produces nitric oxide in an aqueous solution at physiological pH,

which interacts with oxygen to produce nitrite ions, which may be detected using the Griess reagent. Scavengers of nitric oxide compete with oxygen, resulting in reduced nitrite ion formation. Nitrite (NO_2) concentrations beyond a certain threshold can cause tissue damage (Ibrahim *et al*., 2016). In this study, the NO assay was used to evaluate, which fraction had the highest value for suppressing inflammatory activities (Figure 2).

All the extracts were tested for NO scavenging ability, with quercetin as the control. All samples showed dose-dependent inhibitory potential. MFR possessed the strongest anti-inflammatory properties compared to other factions with the lowest IC_{50} value (63.83 ± 0.001 g/ml) as shown in Table 4. The IC_{50} (half-maximal inhibitory concentration) is a the concentration of a compound that is needed to suppress a biological or biochemical process at 50% (Zin *et al*., 2018).

In-vitro Anti-inflammatory Activities

Measurement of Cytotoxicity Activity on RAW 264.7 Cell Lines

MTT assay was used to examine the cytotoxic effect of MFR at concentrations ranging from 7.182 to 1,000 g/mL on RAW 264.7 cells. The cytotoxicity was done on the MFR only since it is proven that this fraction has the highest anti-inflammatory activities in the NO-biochemical assay. The cytotoxic effect of MFR on RAW 264.7 cells was assessed after 24 hours of exposure (Figure 3). The intracellular purple formazan formed by the reduction of MTT solution in the presence of mitochondrial dehydrogenase was used to calculate the percentage of living cells.

According to the US National Cancer Institute, no substantial cytotoxicity was observed: IC_{50}: 20 g/mL = extremely cytotoxic; IC_{50}: 21-200 g/mL = moderately cytotoxic; IC_{50}:

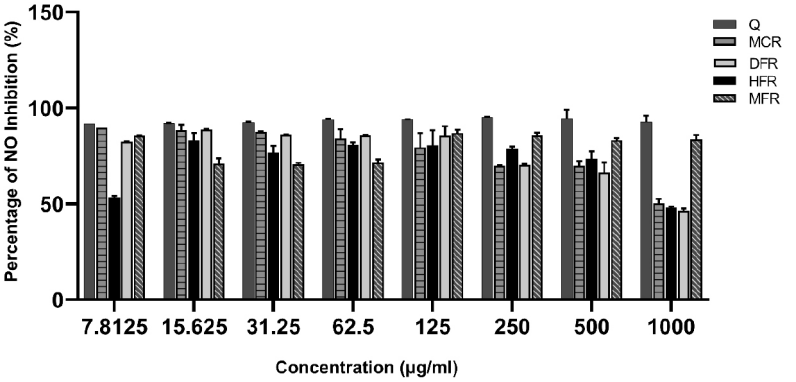

Figure 2: The percentage of anti-inflammatory activities in each sample. The result was expressed as the mean ± SD triplicates

Table 4: The IC_{50} of anti-inflammatory activity value of each fraction of *H. itama* propolis

Samples	IC_{50} µg/mL
MCR	NA
HFR	NA
DFR	NA
MFR	63.83 ± 0.001
Quercetin	40.93 ± 0.003

NA= non-active.

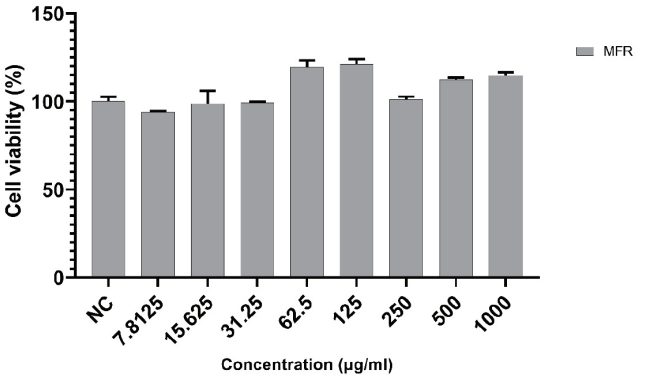

Figure 3: Cell viability percentage for the MFR of *H. itama* propolis

201-500 g/mL = weakly cytotoxic; and IC_{50}: > 501 g/mL = non-cytotoxic (Geran *et al*., 1972). MFR concentrations up to 1,000 g/mL did not display cytotoxicity against on RAW 264.7 macrophage cell lines, these concentrations were used in further investigations.

NO Content in RAW 264.7 Cell Culture Medium

The body's repair mechanism is initiated by a complex network of chemical reactions and cell signalling. The activation and migration of leukocytes (neutrophils, monocytes, and eosinophils) from the venous system to the site of damage, as well as the release of growth factors, cytokines, and reactive oxygen and nitrogen species are all recognised to be important in the inflammatory response (Tolba *et al*., 2013). Propolis has been shown to have anti-inflammatory properties; however, the mechanism is still unknown.

However, prior research indicated that Chinese propolis rich in flavonoids did not affect the viability of RAW 264.7 cells. However, it dramatically reduces the generation of NO, interleukin-1 (IL-1), and interleukin-6 (IL-6) in a dose-dependent manner. The NO inhibitory assay research used various concentrations (0-1,000 µg/ml) (Asgharpour *et al*., 2019).

Macrophages, as the primary line of defence created by monocyte differentiation in response to an infection or wounded cells, play a key role in the process of tissue homeostasis restoration (Asgharpour *et al*., 2019). Therefore, RAW264.7 macrophage cell lines were used in *in-vitro* nitrite oxide assay. This is the most popular cell line utilised in anti-inflammatory research (Lu *et al*., 2020; Jannus *et al*., 2021).

The Griess reaction was used to evaluate the nitrite levels in the media produced by RAW 264.7 cells upon treatment with with LPS (Rao, 2016). LPS is an endotoxin that causes inflammation and can be discovered in Gram-negative bacteria's cytoderm (Zhang *et al*., 2020). Dexamethasone served as the positive control. Sodium nitrite ($NaNO_2$) was used as a standard compound to construct the standard curve (Appendix 3). The NO concentrations were determined by extrapolating the samples' absorbance reading to the standard curve equation, which is $y = 0.9182x + 0.0103$, where x is the NO concentration in µM, and y is the absorbance at 540 nm.

The observed results are significant with $p < 0.001$ compared to NC as shown in Figure 4. The cells were induced with LPS before treatment with MFR. LPS mimics bacterial infection by activating immune cells such as macrophages and causing them to produce nitric oxide (Jannus *et al*., 2021). It also activates microglia to secrete pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), IL-1β, and IL-6, as well as ROS and NO, which can be neurotoxic (Zolfaghari *et al*., 2021). As demonstrated in Table 5, the MFR of *H. itama*

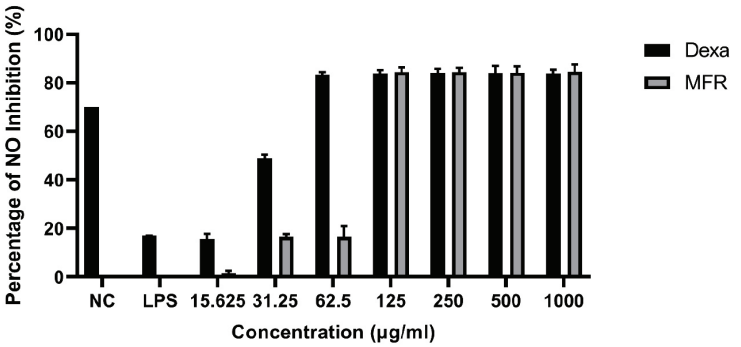

Figure 4: The percentage of nitrite oxide inhibitory activities between dexamethasone and MFR. The result was expressed as the mean ± SD triplicates ($p < 0.0001$) compared to negative control (NC)

Table 5: The IC_{50} for NO inhibition value of dexamethasone and MFR

Samples	IC_{50} value (µg/mL)
Dexamethasone	33.61 ± 0.003
MFR	82.71 ± 0.005

propolis significantly decreased the amount of NO produced by RAW 264.7 cells triggered by LPS with an IC_{50} value of 82.71 ± 0.005 µg/mL, as shown in Table 5. It shows that the MFR of *H. itama* propolis had a strong anti-inflammatory effect via NO-scavenging activity. Based on the result, MFR had a high inhibitory effect on NO production, which may be due to high flavonoid content. The flavonoid component in propolis may downregulate the expression of inducible nitric oxide synthase, which is the responsible enzyme for NO production in inflammatory conditions (Rao, 2016).

The role of nitric oxide in vascular function and inflammation provides insights into how NO production relates to physiological and pathological processes (Gál *et al*., 2023). It is generally recognised that nitric oxide plays a significant role in various inflammatory processes in the animal body. It can be created in significant quantities by iNOS in macrophages. Several agonists can increase iNOS in macrophages, with LPS and cytokines like IFN-γ being the most extensively studied (Hon *et al*., 1997).

A NO test was used in a Saha (2004) study to evaluate the inhibitory effects of different Malaysian medicinal plant extracts. The findings demonstrated that several medicinal plants, including *Chasalia chartacea* and *Lasianthus oblongus* had potent inhibitory effects on the synthesis of nitride oxide. Leukocytes, macrophages, mast cells, platelets, and other immune system components create a range of signalling chemicals that mediate the intricate pathophysiological process of inflammation.

An essential function of macrophages is the production of pro-inflammatory chemicals such as nitric oxide (NO). It has been suggested that NO, produced by the enzyme inducible nitric oxide synthase (iNOS) is a mediator of inflammation and affects both acute and chronic inflammation (Heras *et al*., 2001). After being stimulated with LPS, many cells, including macrophages would express iNOS, which produces significant amounts of NO. This inducible enzyme is a crucial component of inflammation response and is implicated in the pathophysiology of several inflammatory disorders. The Griess test was used to assess the suppression of nitric oxide generation in RAW 264.7 cells produced by LPS and interferon-γ (IFN- γ) (Saha *et al*., 2004).

Conclusions

The results indicate that *H. itama* propolis may possess anti-inflammatory activity, especially in MFR. The data from the phytochemical, biochemical, and *in-vitro* anti-inflammatory tests show that the MFR is non-toxic and possesses a high flavonoid content, which could be responsible for anti-inflammatory activity. Our study may provide scientific evidence for further studies of the stingless bee propolis, especially on the *H. itama* species.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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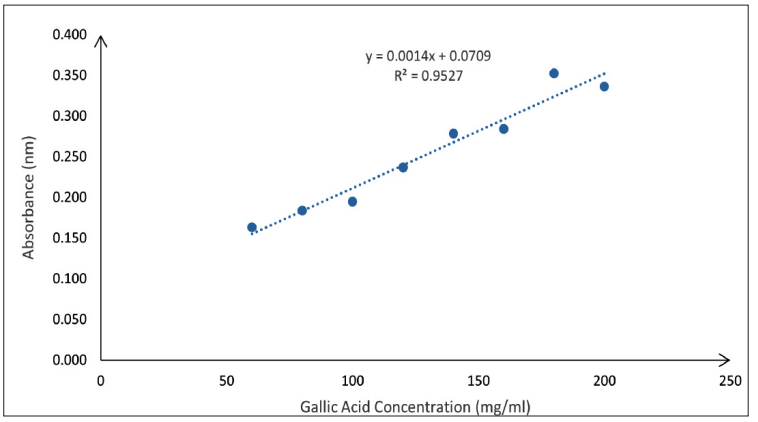
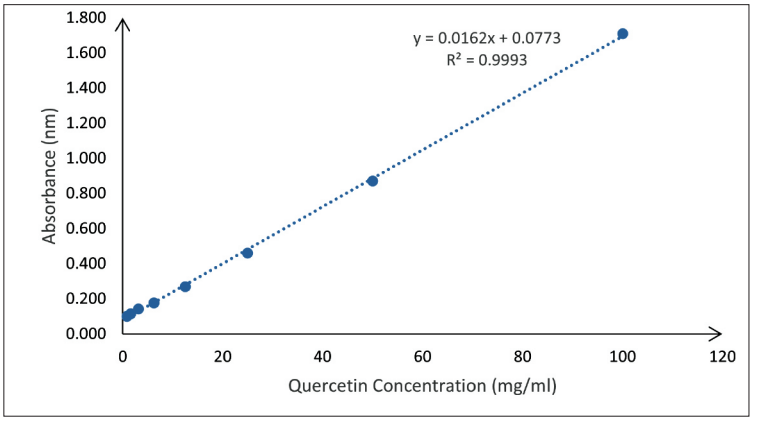
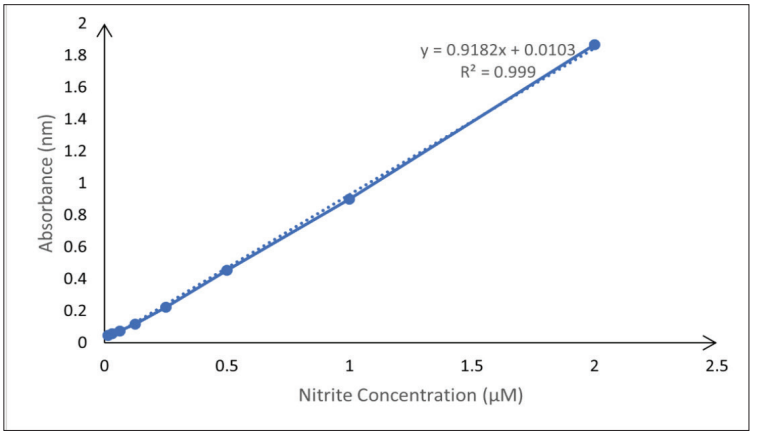
Appendices



Appendix 1: Gallic acid standard curve used in TPC



Appendix 2: Quercetin standard curve used in TFC



Appendix 3: $NaNO_2$ standard curve that is used to obtain NO concentration