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Research article

Phytochemical Analysis and Biological Activities of Propolis from *Geniotrigona thoracica*: Evaluating its Therapeutic Applications

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Abstract: Propolis is a substance that safeguards the bee hive against physical and microbiological threats. This research assessed the phytochemical properties and biological effects of propolis produced by stingless bees *Geniotrigona thoracica* collected from Phatthalung, Southern Thailand. The findings revealed that the ethanolic extract propolis (EEP) from *G. thoracica* exhibited antibacterial properties against certain foodborne pathogens (*Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Typhimurium) with moderate to strong zone of inhibition (ZOI) in the range of 10 mm ≤ ZOI ≤ 15 mm. Additionally, the extract propolis demonstrated antioxidant activity, achieving up to 80% DPPH radical scavenging when 50 mg/mL EEP was tested. Furthermore, the crude propolis extract showed anti-inflammatory effects on macrophage cells, resulting in a 72.9% reduction in nitric oxide (NO) levels in LPS-activated RAW 264.7 cells exposed to 100 mg/mL of EEP. The GC-MS chromatogram identified the phytochemical compositions of the EEP, with Lup-20(29)-en-3-ol or lupeol (25.42%) and β-amyrone (22.66%) as the major compounds, both triterpenoid derivatives. Other notable constituents included alkane hydrocarbon pentacosane (6.63%), fatty alcohol cis-9-eicosenol (4.23%), and phenolic compound 3-pentadecylphenol (3.86%). Therefore, the EEP derived from *G. thoracica*, possessing such diverse biological activities, holds promise for medicinal and functional food applications.

Keywords: Stingless bees; *Geniotrigona thoracica*; Propolis extract; Biological effect

1. Introduction

Stingless bees belong to the Meliponini group, comprising more than 600 species, and are widely distributed in tropical and subtropical regions [1, 2]. Typically, propolis consists of resins collected by bees from plant sources, mixed with saliva and beeswax within the hive. Its primary functions include sealing cracks and protecting against threats [3-5]. The primary component of propolis, comprising over 45%, is lipids, which aid in inhibiting microbial growth. Additionally, propolis contains over 150 compounds, including phenolic

compounds, terpenoids, steroids, and aromatic acids, contributing to its antimicrobial properties. Notably, phenolic compounds such as flavonoids are well-known for their antioxidant properties [6-9]. The bioactive compounds in propolis, especially polyphenols, flavonoids, and terpenes, have led to its application in biomedicine, natural product cosmetics, and as an ingredient in health foods [3, 4, 6, 10-12]. The chemical composition of propolis varies depending on stingless bee species, the timing of collection, the botanical environment, and geographical location [3, 8, 9, 11, 13]**.** Despite the differences in the chemical composition of propolis worldwide, they all show pharmacological activity, making it an attractive natural product. To date, few studies have investigated the chemical composition of propolis from the Thai stingless bee *Geniotrigona thoracica*. Most research has been conducted in Malaysia and Brunei. For example, Nazir et al. [6] identified up to 30 new compounds for the first time from the ethanolic extract of Malaysian *G. thoracica* propolis, with phenolic and terpenoid compounds being the major components as determined by GC-MS analysis [6]. In the propolis of Brunei stingless bees, *G. thoracica* contained lipids as a major component (45.60-47.86%), with minimal carbohydrate and protein content but rich in minerals. Additionally, analysis of functional groups indicates the presence of phenolic and flavonoid compounds. These bioactive compounds contribute to the antioxidant and antibacterial properties of the propolis extract. Ethanol extraction of propolis from *G. thoracica* exhibits antimicrobial effects against both Gram-positive bacteria, e.g., *Bacillus subtilis, Staphylococcus aureus,* and Gram-negative bacteria, e.g., *Escherichia coli, Pseudomonas aeruginosa* [8]. However, the phytochemical compounds, including flavonoid, coumarin, saponin, terpenoid, steroid, and cardiac glycoside, were detected in the ethanol extract of propolis from *G.thoracica*, collected in Pattani province, Thailand, and showing the highest antioxidant activity with the IC_{50} at 262.43 μg/mL and total phenolic content at 60.13 mgGAE/g extract [14]. Therefore, this study aims to investigate the anti-bacterial and antioxidant activity of the ethanolic propolis extract from stingless bees *Geniotrigona thoracica* harvested in Phatthalung Province, Southern Thailand. In addition, the anti-inflammatory potential of its extract is determined by measuring nitric oxide (NO) production. Finally, the bioactive compositions in the propolis extract is identified by GC-MS analysis.

2. Materials and Methods

2.1 Propolis extraction

The propolis sample from *G. thoracica* was collected from Pantae community enterprise, Khuan Khanun district, Phatthalung province, Thailand (locality; 7.806259568725061, 100.01692785193286). The propolis was collected from the beehives between July and September 2023. The collected propolis was rinsed with distilled water and dried using a dehumidifier at room temperature for 2 weeks. After drying, the propolis was ground into small pieces less than 1 millimeter in size. Subsequently, 100 g of dried propolis was extracted in 1 liter of 70% ethanol (the ratio of raw propolis to solvent was 1:10) with an ultrasonicator (DR-MH40, Ultrasonic Cleaner, Derui) for 60 min at 40°C and the sample was then left for maceration for 7 days at 25°C after the treatment. The resulting supernatant was later subjected to rotary evaporation (4001, Heidolph, Schwabach, Germany) until the solvent volume was reduced by approximately half, followed by drying under vacuum at 40°C [7, 8, 12]. The yield content of the propolis extract was calculated by Yield (%) = [Weight of extracted propolis after solvent evaporation(g)/Weight of the initial dried propolis] × 100.

2.2 Antibacterial analysis

The antibacterial activities of the ethanolic extract propolis (EEP) from *G. thoracica* were assessed using the agar disc diffusion assay. The bacterial strains tested included two Gram-positive strains (*Staphylococcus aureus* ATCC-29213 and *Bacillus cereus* ATCC-14579) and two Gram-negative strains (*Escherichia coli* ATCC-11775 and *Salmonella* Typhimurium ATCC 14028). Briefly, the bacterial culture suspension was adjusted in 0.85% (w/v) NaCl to approximately ~10^8 CFU/mL. It was then swabbed on Mueller-Hinton agar, MHA (Oxoid™), while the EEP was prepared to final concentrations ranging from 50 to 1,600 mg/mL. The sterile filter paper discs (6 mm in diameter) containing the extractions were subsequently placed on those MHA. They were

incubated at 37°C for 16-18 hours before assessing bacterial growth inhibition by measuring the diameter of the inhibition zone (mm). Grading of the zone of inhibition (ZOI) followed the description by Bhaigybati et al. (2020) [15], of which 6-8 mm: No antimicrobial activity, 8.1-9 mm: Slight antimicrobial activity, 9.1-12 mm: Moderate antimicrobial activity, 12.1-15 mm: clear antimicrobial activity, and >15 mm: Strong antimicrobial activity. Ampicillin (10 µg) and Ciprofloxacin (5 µg) served as positive controls for Gram-positive and Gram-negative bacteria, respectively, while 5% DMSO was a negative control [13].

2.3 Antioxidant assays

The antioxidant activities of EEP from *G. thoracica* were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [7, 8, 14] by measuring its ability to convert DPPH° into DPPH-H. In brief, 0.1 mL of the extract (ranging from 50 to 200 mg/mL) was combined with 2 mL of DPPH° solution (0.2 mM) in ethanol, and the mixture was left to incubate for 1 hour in darkness at room temperature. The absorbance was then recorded at 517 nm, with ascorbic acid as a positive control. The free radical scavenging activity of the propolis extract was calculated as %Scavenging of DPPH° = $[(A_{\text{Initial Absorbance}} - A_{\text{Final Absorbance}})/A_{\text{Initial Absorbance}}] \times 100$.

2.4 Anti-inflammatory

The level of nitric oxide (NO), a signaling molecule that plays a key role in the pathogenesis of inflammation, of the crude propolis extract from *G. thoracica* was determined using the Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl) ethylenediamine, each in 2.5% H_3PO_4) as described by Mendez-Encinas et al.(2023) [16]. The RAW 264.7 macrophage cell line (ATCC Number: TIB-71™ ,Lot Number: 7006149 ,Species: Nouse (*Mus musculys*))was purchased from Thermo Fisher Scientific Inc., USA. The cells (1×10^5 cells/well, 100 µL) were plated in a 96-well plate and incubated for 24 hours at 37°C and 5% CO_2. Following incubation, the cells were treated with 50 µL of propolis extract ranging from 50 to 800 mg/mL in DMEM and stimulated with 50 µL of 10 µg/mL lipopolysaccharide (LPS), Sigma-Aldrich, USA, in DMEM, then further incubated for 24 hours. Control groups included cells treated with DMSO (negative control), cells treated with LPS (positive control), and cells treated with neither DMSO nor LPS. After incubation, aliquots (50 µL) of cell supernatants were collected, mixed with an equal volume of Griess reagent, and incubated in the dark at room temperature for another 10 min before measuring the absorbance change at 540 nm. Results were expressed as reduced sodium nitrite concentration (µM), then converted to nitric oxide (NO) production.

2.5 Identification and quantification of bioactive compounds in the propolis extract

Gas chromatography/mass spectrometry (GC-MS) analysis was conducted using a GC7890B and MSD5977B system (Agilent Technologies, USA) equipped with an HP-5Ms column (15 m × 250 mm × 0.25 µm). Helium served as the carrier gas at a flow rate of 1 mL/min. The GC-MS condition was as follows: the injector temperature was set at 280°C in split-less mode, the oven temperature was initially maintained at 60°C for 4 min, then increased to 150°C at a rate of 10°C/min for 15 min with a scan range of 35-500 Da [17]. The mass spectra were then compared to the National Institute of Standards and Technology (NIST) library data to identify and quantify the bioactive compounds.

2.6 Statistical analysis

All the values were presented as the mean ± standard deviation (SD). Data underwent analysis via one-way ANOVA, and differences between means were assessed using the LSD multiple range test ($p \leq 0.05$) with the SPSS program (IBM SPSS Statistics, version 27.0.1).

3. Results and Discussion

3.1 Propolis extraction and yield content

The extraction of propolis from *G. thoracica* using 70% ethanol resulted in a yield of approximately 27.21% of the raw sample. The process of preparing the crude extract is illustrated in **Figure 1**. Additionally, the literature suggests that ethanolic extracts contain more aromatic compounds than water extracts [7, 14]. Like the propolis extract from *Cerana indica*, the ethanolic extract had stronger antimicrobial activity than the

methanolic extract and aqueous extract [17]. This might be attributed to its less lipophilic behavior, as many of the phytochemicals possess electronegative functional groups, rendering them hydrophilic. The secondary metabolites are also highly soluble in organic solvents [6, 18]. Nevertheless, the maceration method using organic solvents is extensively utilized for propolis extraction. Indeed, maceration with 70% ethanol is the preferred organic solvent for propolis extraction [5, 18]. For instance, Silva et al. (2012) conducted a study showing variations in extraction efficiencies among different organic solvents. They found that the hydroalcoholic-extracted propolis demonstrated the highest propolis and flavonoid content levels of about 280 mg and 140 mg, respectively [19].

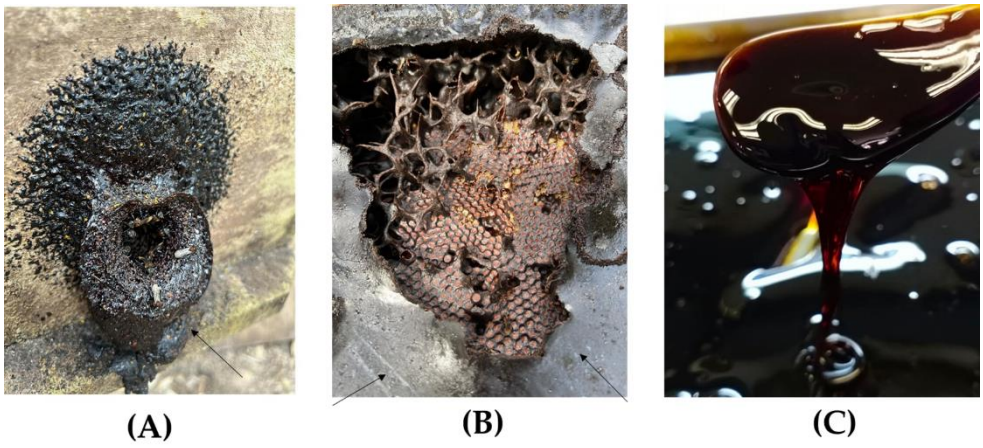


Figure 1. Propolis of *G. thoracica* collected in Pantae community enterprise, Khuan Khanun district, Phatthalung province, Southern Thailand. (A) the entrance of the *G. thoracica* colony and its adults, (B) raw propolis (with the arrows pointing), and (C) the ethanolic extract propolis (EEP) of *G. thoracica*

3.2 Anti-bacterial Activity

The anti-microbial properties of the EEP from *G. thoracica* against certain important foodborne pathogens, including two Gram-positive bacteria (*B. cereus* and *S. aureus)* and two Gram-negative bacteria (*E. coli* and *S.* Typhimurium) was, examined by disc diffusion assay to qualify its inhibitory activities on the tested strains. The inhibition zone of the various crude EEPs was demonstrated in **Table 1**.

Table 1. Antibacterial activity of crude propolis extract of *G. thoracica* against different foodborne pathogens

Antibacterial activity	Concentration	Inhibition zone (mm± SD)			
		B. cereus	*S. aureus*	*E. coli*	*S.* Typhimurium
Ethanolic extract propolis (mg/mL)	50	15.0 ± 0.00	13.5 ± 1.50	10.0 ± 0.01	10.0 ± 0.01
	100	15.5 ± 0.50	12.5 ± 0.50	10.0 ± 0.01	10.0 ± 0.01
	200	15.5 ± 0.50	13.0 ± 0.02	10.0 ± 0.02	11.0 ± 0.04
	400	15.0 ± 0.02	14.0 ± 0.02	-	11.0 ± 0.02
	800	14.5 ± 0.50	12.0 ± 0.05	-	-
	1,600	-	-	-	-
Positive control	Ampicillin[a] (10 µg)	35.0 ± 0.00	35.0 ± 0.02	-	-
	Ciprofloxacin[b] (5 µg)	-	-	30.0 ± 0.01	30.0 ± 0.01
Negative control	5% DMSO	-	-	-	-

(-) refer No inhibition where 6-8 mm: No antimicrobial activity, 8.1-9 mm: Slight antimicrobial activity, 9.1-12 mm: Moderate antimicrobial activity, 12.1-15 mm: clear antimicrobial activity, and >15 mm: Strong antimicrobial activity.

[a] Ampicillina used as a positive control for Gram-positive bacteria

[b] Ciprofloxacin used as a positive control for Gram-negative bacteria

Mean values of three replicates ± standard deviation (SD)

The results revealed that the EEP from *G. thoracica* showed good dose-dependent antibacterial activity. The highest growth inhibition was obtained in the 200 mg/mL extract sample with the largest zone of inhibition (ZOI) of about 15.5 ±0.50 mm against *B. cereus*, while in *S. aureus* needed about 400 mg/mL of the propolis extract to achieve the highest ZOI of 14±0.02 mm. On the other hand, in the tested Gram-negative bacteria (*E. coli* and *S.* Typhimurium), the crude extract showed deficient inhibition (an average ZOI of 10-11 mm). These bacterial inhibition tests of the EEP from *G. thoracica* were graded as moderate to strong antimicrobial activity. It is worth understanding that all these bacteria tests are considered foodborne diseases. In addition, *S. aureus* and *Salmonella* spp. were on the Thai Agricultural Standard Tas 8003-2013 list for honey. Abdullah et al. (2020) evaluated the potential of 2 mg/mL ethanol extract propolis from three different stingless bee species, *Geniotrigona thoracica, Heterotrigona itama,* and *Tetrigona binghami. S*imilarly to this study, *G. thoracica* propolis demonstrated the highest ZOI against *E. coli*, followed by *B. subtilis*, *P. aeruginosa*, and *S. aureus*, respectively, while in *H. itama* propolis showed the largest clear zone among this three propolis with approximately 13 mm against *B. subtilis* [8]. On the other hand, the investigation of the EEP from *G. thoracica*, *T. binghami*, and *H. itama* carried out by Zullkiflee et al. (2022) revealed that the highest ZOI of 11.7 mm obtained in *G. thoracica* propolis against *S. aureus,* in addition, the overall antibacterial inhibition was higher potent in the ethanolic extracts than in water extract. The MIC was in the range of 2,500-10,000 μg/mL[7]. Similar trends were found in the extract of Malaysian propolis produced by *H. itama* and *G. thoracica,* which inhibited the growth of *S. aureus* better than Gram-negative (*E. coli* and *Salmonella typhi*). The variability in antibacterial activity against different bacterial strains is attributed to the diversity of bioactive compounds in propolis from various species of stingless bees. Literature suggests that the antimicrobial properties of propolis are related to phenolic and flavonoid compounds of varying polarities and their synergistic effects. These polar and lipophilic compounds, containing electronegative functional groups, e.g., carbonyl, amine, thiol, or hydroxyl groups, interacted with the cell wall and membrane of bacterial cells, resulting in the leakage of cellular components and, finally, cell death [3-5, 10, 11, 20]. In general, Gram-negative bacteria are more resistant to antibacterial agents than Gram-positive bacteria due to the presence of the outer membrane, which is comprised mainly of LPS that make Gram-negative bacteria more invulnerable to the antimicrobial agents as well as the function of the efflux pumps [21, 22]. In addition, the extracts from the propolis efficiently inhibited fungal species such as *Candida albicans* and *C. neoformans* with a MIC of 1.56 mg/mL while in stingless bee *Melipona beecheii* was able to inhibit the growth of *C. albicans* and induced dramatic changes in the structure and integrity of the cell wall [23]. A similar observation also found in propolis from *Tetragonisca fiebrigi* that was able to suppress the growth of *C. albicans* and *C. glabrata* [12]. This antifungal activity may also be provided by its phenolic and flavonoid compounds [24]. Similarly, results of antibacterial effect were observed in Malaysian *G. thoracica* honey, with the inhibition zones about 9 -12 mm for the tested population against *S. aureus* and *E. coli*, while the *G. thoracica* honey from Borneo (Sarawak) showed antibacterial against Gram negative bacteria which was in the range of ZOI about 12.3±0.21- 30±0.10 mm [25]. The antimicrobial efficacy observed in stingless bee propolis may be credited to the actions of flavonoids and other chemical constituents. Moreover, factors such as the extraction method, osmotic effect, or the properties of phytochemicals may also contribute to the antimicrobial activity of the propolis produced by stingless bee [3, 26].

3.3 antioxidant Activity

The scavenging potential of the propolis extracts against DPPH° is presented in **Figure 2**. DPPH° undergoes a conversion to DPPH-H upon accepting a hydrogen atom from phenolic compounds. The phenolic compound increases directly with the intensity of DPPH° [14,26,27]. The assessment demonstrated that the % DPPH scavenging was increased by increasing the propolis extract concentration. However, the more EEP added, the more the antioxidant activity maintained at approximately 80% radical scavenging activity, accounting for about 0.8 times of ascorbic acid. Comparing to a study carried out by Akhir et al. (2018) found that the propolis extract from *H. itama* in ethanol-solvent produced a higher antioxidant activity than in n-hexane and also showed a strong positive correlation with total phenolic and flavonoid content [26,28]. The potent antioxidant properties are related to the chemical composition of the propolis [3-5,11]. Furthermore, the antioxidant capacity of propolis produced by *G. thoracica, H. itama*, and *T. binghami* collected in Brunei,

which was extracted in ethanol, revealed varying total antioxidant capacities (TAC) with the highest TAC observed in *H. itama* (317.6 mgAAE/g), followed by *G. thoracica* (42.5 mgAAE/g) and *T. binghami* (12.3 mgAAE/g). In addition, the ethanol extract of propolis from *G. thoracica*, collected in Pattani province, Thailand, exhibited flavonoid, coumarin, saponin, terpenoid, steroid, and cardiac glycoside constituents, demonstrating an IC_{50} value of 262.43 μg/mL and a total phenolic content of 60.13 mg GAE/g extract [11]. Obviously, according to Figure 2 in this study, after the percentages of DPPH radical scavenging were calculated as IC_{50}, the results showed the IC_{50} of approximately 20 mg/mL obtained in EEP from *G. thoracica* while in ascorbic acid reached the IC_{50} less than 12.5 mg/mL. This obtained IC_{50} was almost similar value to *G. thoracica* ethanolic extract propolis that was collected in Serdang, Selangor, Malaysia (N 2° 58′ 45.84″ E 101° 41′ 51.72″), predominantly surrounded by medicinal plants from the *Simaroubaceae*, *Myrsinaceae*, *Primulaceae*, *Zingiberaceae*, *Acanthaceae* and *Lamiaceae* families [9]. Phenolic compounds and flavonoids found in plant constituents are recognized as potent free radical scavengers [27], indicating the significant role of phenolic compounds in the antioxidant activity of the propolis extract. Moreover, Idris et al. (2023) confirmed a strong correlation between total phenolic content (TPC), total flavonoid content (TFC), and IC_{50} of DPPH, indicating that the radical scavenging activity of propolis extract is influenced by the phenolic and flavonoid contents owing to the presence of aromatic hydroxyl groups, which are known for their effective electron accepting abilities [9].

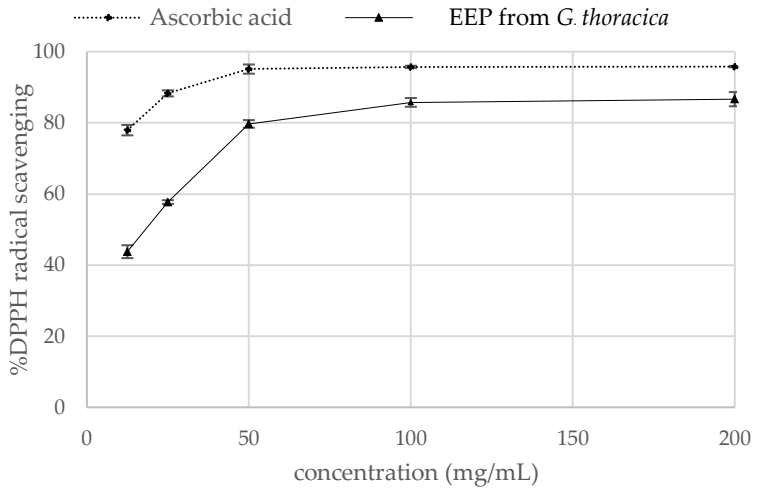


Figure 2. %DPPH° radical scavenging of the ethanolic propolis extract (EEP) of *G. thoracica* (ascorbic acid was used as a positive control) Values represent means ± SD of three independent experiments.

3.4 Anti-inflammatory Activity

NO inhibitory assay was used to test for anti-inflammatory properties of the propolis extract [17, 29]. This study demonstrated the NO production in RAW 264.7 macrophage cells treated with EEP from *G. thoracica* **(Figure 3)**. Interestingly, the anti-inflammatory activity of *G. thoracica* propolis in Thailand has not been investigated much. This study evaluated the ethanolic extract from *G. thoracica* propolis collected in Phatthalung province area by measuring NO production. In this case, LPS is used as an inflammation inducer to induce inflammation in RAW 264.7 cells. It stimulates the cells to produce pro-inflammatory cytokines and mediators, creating an inflammatory environment. Therefore, it is hypothesized that the LPS-induced inflammatory response decreases the levels of inflammatory markers such as NO production, indicating the anti-inflammatory activity of the EEP.

The results showed that at the lowest concentration tested (100 mg/mL), NO secretion in RAW 264.7 cells was reduced to basal levels, decreasing NO production by 72.9% in LPS-activated cells. However, increasing the EEP concentration from 200 to 800 mg/mL appeared to raise nitrite levels, which could potentially trigger an excessive inflammatory response. These may suggest that 100 mg/mL of EEP showed the most substantial reduction in nitrite concentration, indicating a strong anti-inflammatory effect at this

concentration. Still, for a precise IC_{50}, a consistent dose-response curve between 0 and 100 mg/mL is required. However, it can be explained by the non-linear relationship between propolis extract dosage and nitrite production. Lower doses of the extract might exhibit anti-inflammatory effects, while higher doses could potentially induce a pro-inflammatory response, resulting in increased nitrite production [30].

Similarly, the previous study in Brazilian red propolis showed that 50 μg/mL of propolis extract decreased NO production by 78% in LPS-activated RAW 264.7 macrophages cells [31] while in Sonoran propolis at a concentration of 10 μg/mL was able to decrease NO level between 86% and 95% [16]. These results suggested that propolis is supposed to be anti-inflammatory by inhibiting NO production in macrophages [16, 29]. NO is a signaling molecule in the inflammation process and is naturally produced in biological tissues. This may explain why propolis could reduce the levels of specific molecules, such as hydroxyarginine, an intermediate molecule in NO production [31, 32]. The anti-inflammatory mechanism of propolis is associated with its intricate chemical composition. Regardless, the propolis extract's phenolics and flavonoids are considered anti-inflammatory agents. For example, the flavonoids can inhibit the enzyme-inducible NO synthase (iNOS) by binding to the PPAR-γ receptor on macrophage cells [16, 30-33]. However, further studies may include a positive control such as Dexamethasone, a known standard for suppressing the expression of pro-inflammatory cytokines, e.g., TNF-α, IL-6, and IL-1β and certain enzymes such as iNOS and COX-2 by inhibiting transcription factors [34], to quantify the exact efficacy of the EEP affect on anti-inflammatory of the treated cells.

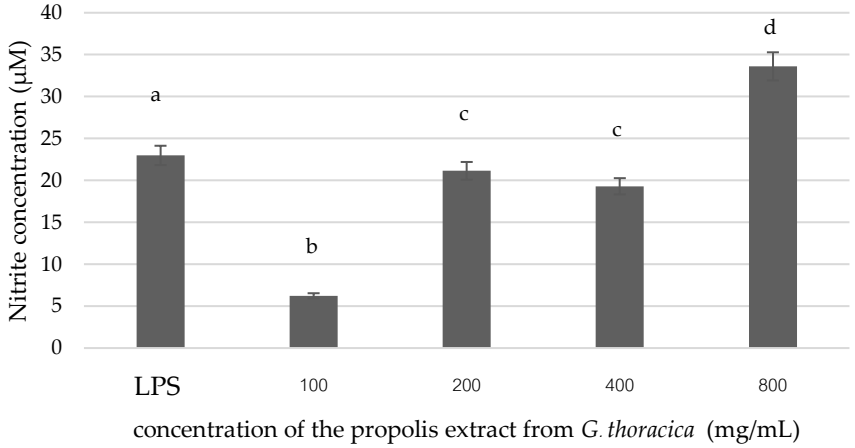


Figure 3. Effect of the ethanolic propolis extract of *G. thoracica* on nitrite levels (LPS was used as an inflammation inducer). Bars with different letters within the same concentration group indicate statistical differences ($p \leq 0.05$).

3.5 Identification and quantification of bioactive compounds in the propolis extract

The chemical constituents found in various types of stingless bee propolis primarily consist of phenolic and flavonoid compounds such as quercetin, vanillic acid, coumaric acid, and benzoic acid [3, 6, 8, 10, 12]. Phenolics are compounds developed by the secondary metabolism of plants [3, 8, 27]. Notably, there were few studies of the chemical composition of the propolis from *G. thoracica*. For example, Nazir et al. [6] studied the chemical constituents of *G. thoracica* propolis in Malaysia and successfully identified 30 new compounds from the ethanolic extract of propolis. While in Brunei, *G. thoracica* propolis contained aromatic acids, terpenes, flavonoids, and phenolic acids with hydroxyl functional groups based on FTIR analysis [6]. The highlights of the phytochemical compounds in the EEP from *G. thoracica* include the triterpenoid derivatives lupeol (25.42%) and β-amyrone (22.66%) as the dominant compounds. These are followed by pentacosane (6.33%), cis-9-eicosenol (4.23%), and the phenolic compound phenol 3-pentadactyl (3.86%), as presented in **Table 2**. Lupeol is a pentacyclic triterpenoid commonly found in the plant. It is used to reduce

inflammatory responses and has immunomodulating properties. Lupeol and its derivatives have a great potential to act as an inflammatory, anti-microbial, and anti-protozoal. Various studies have shown that the anti-inflammatory activity of lupeol through the modulation of p-38 pathways inhibits inflammation[35]. These results validate the importance and role of terpenes in providing bioactive properties to the composition of propolis. The Triterpenoids have been studied for their ability to regulate immune responses, reduce inflammation, and protect the cells from damage caused by oxidative stress [3-5, 16, 19], while the phenolic compounds, including flavonoids and phenolic acids, are known for their antioxidant properties, reducing inflammation, and modulating cellular signaling pathways [8-11, 30]. Although phenols and terpenes are commonly present in many types of propolis, their concentrations, proportions, and types differ among propolis varieties. These variations can be attributed to various extrinsic and intrinsic factors, including botanical sources, geographical location, extraction techniques, climate conditions, bee species, and the foraging preferences of each bee species [3, 5, 6, 12, 17]. Similar results to Nazir et al. [6] reported the EEP from Malaysian *G. thoracica* consisted of 1H-Pyrrole-2-carboxylic acid and 1-(2-hydroxy-2-phenylethyl) as major phenolic compounds followed by terpenoid and its derivatives [6].

Table 2. Phytochemical compounds in the ethanolic extract propolis of *G. thoracica* detected by GC-MS analysis

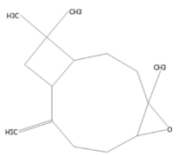
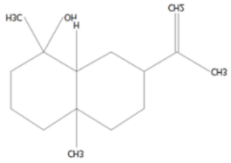
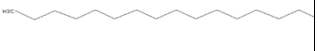
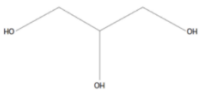
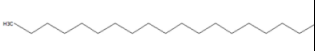
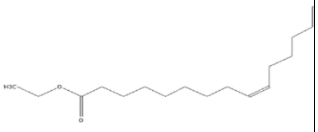
Retention time (min)	Compound Names	Molecular structure	Classification of Phytochemicals	% of total component area*
17.4939	(-)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,12- trimethyl-9-methylene-[1R- (1R*,4R*,6R*,10S*)]-	 $[C_{15}H_{24}O]$	tricyclic diterpenoids	1.04
23.5475	Neointermedeol	 $[C_{15}H_{26}O]$	sesquiterpene alcohol	1.35
23.8899	Hexadecanoic acid, ethyl ester	 $[C_{18}H_{36}O_2]$	ethyl ester	2.67
24.711	Glycerol	 $[C_3H_8O_3]$	polyol compound	1.36
24.8731	Tricosane	 $[C_{23}H_{48}]$	alkane hydrocarbon	1.00
27.9522	9-Octadecenoic acid (Z)-, ethyl ester	 $[C_{20}H_{38}O_2]$	ethyl ester	2.49

Table 2. Phytochemical compounds in the ethanolic extract propolis of *G. thoracica* detected by GC-MS analysis **(Continue)**

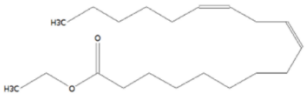
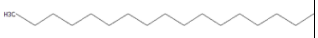
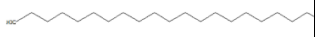
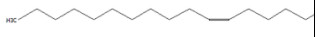
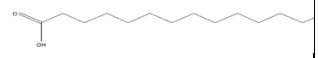
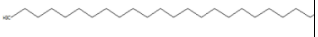
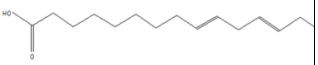
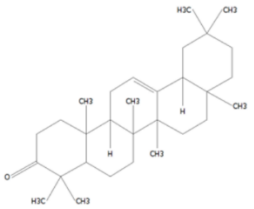
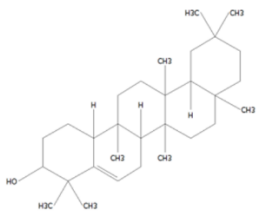
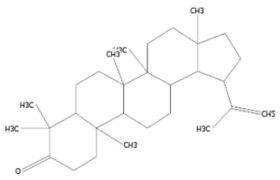
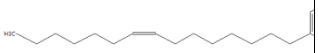
Retention time (min)	Compound Names	Molecular structure	Classification of Phytochemicals	% of total component area*
28.7355	Linoleic acid, ethyl ester	 [$C_{20}H_{36}O_2$]	ethyl ester	2.63
29.6809	1-Octadecanol	 [$C_{18}H_{38}O$]	fatty alcohol	1.47
31.8903	Pentacosane	 [$C_{25}H_{52}$]	alkane hydrocarbon	6.63
34.1700	Eicosen-1-ol, cis-9-	 [$C_{20}H_{40}O$]	fatty alcohol	4.23
36.5174	Palmitic acid	 [$C_{16}H_{32}O_2$]	fatty acid	1.63
36.8494	Nonacosane	 [$C_{29}H_{60}$]	alkane hydrocarbon	1.92
44.4285	Linoleic acid	 [$C_{18}H_{32}O_2$]	fatty acid	1.77
48.0478	.beta.-Amyrone	 [$C_{30}H_{48}O$]	triterpenoid derivative	22.66
51.2512	Glutinol	 [$C_{30}H_{50}O$]	triterpene alcohol	1.37
54.7193	Phenol, 3-pentadecyl-	 $C_{21}H_{36}O$	phenolic compound	3.86

Table 2. Phytochemical compounds in the ethanolic extract propolis of *G. thoracica* detected by GC-MS analysis (Continue)

Retention time (min)	Compound Names	Molecular structure	Classification of Phytochemicals	% of total component area*
55.4216	Lup-20(29)-en-3-one	 $C_{30}H_{48}O$	triterpenoid derivative	25.42
56.8207	(Z)-3-(pentadec-8-en-1-yl)phenol	 $C_{21}H_{34}O$	phenolic compound	2.35

* The GC-MS analysis data here presented only the compound content ≥1 % of the total component area

In addition, according to an observation of major plants and pollens in the propolis collection site, e.g., Pantae community enterprise, Khuan Khanun district, Phatthalung province, Thailand, it was found that the oil palm tree *(Elaeis guineensis)* and the rubber tree *(Hevea brasiliensis)* were dominant plant species. These results corresponded with the phytochemical compounds described above, especially the triterpene derivatives derived from isoprene units obtained from the latex, bark, leaves, or other parts of the rubber tree plant. Although the stingless bee species are similar, the plant sources surrounding them may differ, resulting in the bioactives' variation [3, 5]. In addition, a small amount of fatty acid compounds, e.g., linoleic acid and palmitic acid, were also identified in this study. In summary, the chemical composition of stingless bee propolis comprises aromatic acids, phenolic compounds, alcohols, terpenes, and sugar as the dominant compounds [3, 5, 13 ,28]. According to the literature reviews, up to 16 species of stingless bee-producing propolis that were harvested in Malaysia, Brazil, Mexico, Thailand, the Philippines, Vietnam, and India reveal the largest amount of phenolic compounds (e.g., *p*-coumaric acid and gallic acid). However, the chemical compositions vary significantly when comparing propolis from the same species of stingless bees. These differences are due to variations in the identification methods, collection locations, and collection periods for the propolis [3, 5]. The hypothesis suggests that the bioactive compounds present in propolis may interact synergistically, combining their complementary mechanisms of action to enhance the beneficial biotherapeutic potential of this valuable bee product [3-5,9]. The synergistic effects between the various bioactive components in propolis often occur. For example, antibacterial synergy by different compounds in propolis may target different aspects of bacterial physiology, such as lupeol. It can integrate into the bacterial cell membrane, causing disruption of its integrity and has been shown to inhibit certain bacterial enzymes, in addition, can interfere the biofilm formation [35, 36], while β-amyrone may inhibit the synthesis of bacterial cell walls and also can affect key metabolic pathways in bacteria including the inhibition of the enzymes lipase, α-glucosidase, and α-amylase, disrupting their growth and replication [37]. Therefore, both Lupeol and β-anyone may have a synergistic effect as their different mechanisms of action can target multiple bacterial processes simultaneously. Additionally, both compounds in EEP contribute antioxidant activity by neutralizing free radicals and reducing oxidative stress. Notably, β-Amyrone may influence endogenous antioxidant enzyme activity to further enhance its antioxidant potential [38]. Finally, these triterpenoid derivatives revealed capability of interacting with multiple molecular targets, affecting and modulating the inflammation process, carcinogenesis and cellular stress response by inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6,and may suppress the activation of NF-κB signaling pathway, which is a central regulator of inflammation, resulted in lower expression of inflammation-related genes [30, 36, 37]. Therefore, the extracted propolis acts through a combination of

mechanisms to exert its antibacterial, antioxidant, and anti-inflammatory effects, primarily due to its rich content of bioactive compounds such as triterpenoid derivatives as mentioned above.

4. Conclusions

This study unveils the phytochemical composition of ethanolic extract propolis (EEP) sourced from *G. thoracica*, harvested in Phatthalung province, Southern Thailand, revealing its significant influence on biological activities such as antibacterial, antioxidant, and anti-inflammatory capabilities. The primary constituents found in the EEP were triterpenoids and phenolic compounds, recognized for their potential as bioactive. Remarkably, the EEP exhibited remarkable outcomes, including up to 80% DPPH radical scavenging activity and a notable 73% reduction in NO production in LPS-activated RAW 264.7 macrophage cells, accompanied by reduced anti-inflammatory effects and robust antibacterial activity with moderate to strong inhibition. These findings position the propolis of *G. thoracica* as a promising therapeutic agent and a safe food supplement. Additionally, this study offers novel insights into the phytochemical and biological activities of extracted propolis from the Phatthalung region of Thailand.

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