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BIOASSAY-GUIDED FRACTIONATION OF POTENTIAL ANTIOXIDANT CONSTITUENTS FROM *Geniotrigona thoracica* PROPOLIS AND IDENTIFICATION OF ITS FLAVONOID COMPOUNDS VIA Q-TOF LCMS

(Pengasingan Sebatian Antioksidan Berpotensi Berpandukan Pencerakinan Biologi daripada Propolis *Geniotrigona thorasica* dan Pengenalpastian Sebatian Flavonoid Menggunakan Q-TOF LCMS)

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Abstract

Recently, stingless bee propolis has gained attention in the food market, mainly due to its pharmacological properties. Previous studies have reported the higher antioxidant properties of *Geniotrigona thoracica* propolis extract. In this paper, we report on the bioassay-guided fractionation of the active *n*-hexane fraction of *G. thoracica* propolis and its flavonoid compounds by Quadrupole-Time of Flight Liquid Chromatography Mass Spectrometry (Q-TOF LCMS) analysis. Chromatographic separation of *n*-hexane fraction was carried out using column chromatography. Antioxidant activities were evaluated using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and 2,2'-azinobis (3- ethylbenzthiazolin-6-sulfonic acid) (ABTS) free radical scavenging assays. The identification of flavonoid compounds was carried out using Q-TOF LCMS analysis. Eleven sub-fractions resulting from the chromatographic separation of *n*-hexane fraction showed DPPH and ABTS radical scavenging activity with IC_{50} values ranging from 468.60 to 5.83 µg/mL and 248.30 to 4.00 µg/mL, respectively. A total of seven flavonoid, phenolic and anthocyanin compounds were identified such as naringenin (**1**), acacetin (**2**), eriodictyol (**3**), myricetin (**4**), chlorogenic acid (**5**), eriodictyol-7-O-glucoside (**6**) and cyanidin chloride (**7**). Most of these compounds have been reported to possess antioxidant activity in previous studies. This indicate that *G. thoracica* propolis has potential source of antioxidants for the development of food and nutraceutical products.

Keywords: *Geniotrigona thoracica*, stingless bee propolis, bioassay-guided fractionation, flavonoid, Q-TOF LCMS

Abstrak

Kebelakangan ini, propolis lebah kelulut telah menarik perhatian di dalam pasaran makanan, terutamanya disebabkan oleh sifat famarkologi yang dimilikinya. Kajian terdahulu melaporkan sifat antioksidan yang tinggi untuk estrak propolis *Geniotrigona thorasica*. Dalam kajian ini, kami melaporkan pengasingan sebatian fraksi aktif *n*-heksana propolis *G. thorasica* dan sebatian flavonoidnya melalui analisis Spektrometri Jisim Kromatografi Cecair Penerbangan- Empat Masa (Q-TOF LCMS). Pemisahan kromatografi fraksi *n*-heksana dilakukan menggunakan kromatografi lajur. Aktiviti antioksidan dinilai menggunakan ujian pembebasan radikal bebas 1,1-difenil-2-pikrilhidrazil (DPPH) dan 2,2'-azino-bis (3-etilbenzotiazolin-6-sulfonik asid) (ABTS)). Pengenalpastian sebatian flavonoid dilakukan menggunakan analisis Q-TOF LCMS. Sebelas sub-fraksi dihasilkan daripada pemisahan kromatografi di dalam fraksi *n*-heksana telah menunjukkan aktiviti pembebasan radikal bebas DPPH dan ABTS dengan

nilai IC_{50} masing-masing berjulat antara 468.60 sehingga 5.83 µg/mL dan 248.30 sehingga 4.00 µg/mL. Sebanyak tujuh sebatian flavonoid, fenolik dan antosianin telah dikenalpasti seperti naringenin (**1**), asetin (**2**), eriodiktiol (**3**), mirisetin (**4**), asid klorogenik (**5**), eriodiktiol-7-O-glukosida (**6**) dan klorida sianidin (**7**). Kebanyakan sebatian ini telah dilaporkan memiliki aktiviti antioksidan di dalam kajian terdahulu. Ini menunjukkan bahawa propolis *G. thorasica* merupakan sumber potensi antioksidan untuk pembangunan produk makanan dan nutraseutikal.

Kata kunci: *Geniotrigona thorasica*, propolis lebah kelulut, pencerakinan berpandukan biologi, flavonoid, Q-TOF LCMS

Introduction

Stingless bees (Hymenoptera; Apidae; Meliponini) belong to eusocial bees and are close relative to honeybees, *Apis mellifera*. They are widely distributed in tropical and subtropical forests with warm and humid environments [1,2]. The name 'stingless' emerged due to the significant reduction in their sting and that they defend their nest by biting [3]. To date, more than 60 genera and 600 species have been identified across various countries such as Neotropics (South and Central Americas), tropical Africa, Australia and Southeast Asia [4]. Stingless bees also known as *Kelulut* bees, are among the native bee species in Malaysia that serve as important pollinators for crops in the agriculture industry. In Malaysia, more than 35 species were identified and it is expected new species will be discovered in the future. The most important species are *Heterotrigona itama*, *Geniotrigona thoracica*, *Tetragonula laeviceps*, *Lepidotrigona terminata* and *Tetrigona apicalis* [5].

Propolis, is one of the by-products of stingless bees aside from honey and bee bread. It is a resinous material composed of plant resin, beeswax and latex [6]. Bees utilize propolis to build internal walls, protect the hive from diseases, and cover intruders' carcasses, avoiding their decomposition [7]. The researchers proved that the chemical constituents of propolis depends on several factors, such as plant sources, geographical origins, bee species and time of propolis collection [8,9]. *G. thoracica* propolis was reported to contain triterpenes, oleyl alcohol, 2-methoxy-4-vinylphenol, and (9Z)-octadeca-9,17-dienal [10]. Other phytochemicals identified in *G. thoracica* propolis include phenol, benzoic acid, trimethylsilyl ester, hydroginkgol, resorcinol, delta-cadinene, nootkatone, *β*-amyrenol, friedelanyal, cycloeucaleno, myristic acid, palmitic acid, linoleic acid, octadecanoic acid [11], lanosterol, *β*-amyrin, *α*-amyrin 13,2-cycloursan-3-one, 9,19-cyclolanost-24-en-3-ol, 24-methylene, and 9,19-cyclolanost-24-en-3-ol [12].

Propolis possesses many biological and pharmacological activities, such as antioxidant, antibacterial, anticancer, anti-inflammatory, and antidiabetic [13-15]. The bioactivities of *G. thoracica* propolis include antioxidant, antimicrobial, anticancer, and anti-atherosclerosis [10,16-18]. Based on the previous findings, *G. thoracica* propolis was found to possess higher antioxidant ability. In this study, a bioassay-guided fractionation approach was carried out using DPPH and ABTS assays to identify the active fractions of *G. thoracica* propolis. The identification of the flavonoid compounds from the active fractions was then conducted using Q-TOF LCMS analysis. A total of five flavonoid compounds were identified which indicate the active compounds for antioxidant activity. To the best of our knowledge, this is the first report on the identification of these antioxidant constituents from the *G. thoracica* propolis extract.

Materials and Methods

General experimental procedures

Thin layer chromatography (TLC) was performed using silica gel 60 F_{254}, precoated with 0.2 mm aluminium plates (Merck, Germany). Spots were detected using UV light of 254 and 366 nm followed by derivatization using ferric chloride reagent (Merck, Germany). Column chromatography (CC) was performed using silica gel 60 (0.040-0.063 mm, Merck, Germany). Quadrupole Time of Flight LCMS (Vion IMS LCQTOF MS) from Waters Corporation and C18 ACQUITY UPLC-HSS T3 column (2.1 mm x 100 mm x 1.8 µm) were used for the LCMS analysis. The identification of compounds was conducted using ReSPect (RIKEN MSn spectral database for phytochemicals) and comparison with literature. All solvents used for chromatography were of high-performance liquid chromatography (HPLC) grade

while the other chemicals were of analytical reagent grade. Quercetin and trolox (Merck, Germany) were used as positive control for the DPPH and ABTS assays.

Extraction and fractionation of propolis

Fresh *G. thoracica* propolis was collected at Apiary Farm, Universiti Sultan Zainal Abidin, Besut, Terengganu, Malaysia. Propolis was stored at -80°C prior to extraction. Approximately 500 g of powder propolis was macerated with 1.2 L of methanol for three days to yield methanolic crude propolis extract (311.79 g). Methanol was chosen as the extraction solvent following its ability to dissolve a wide range of bioactive compounds. The fractionation of crude propolis extract was carried out using the trituration method [19] with *n*-hexane (*n*-Hxn) and dichloromethane (DCM) to yield 43.99 g of *n*-Hxn fraction and 145.86 g of DCM fraction, respectively.

Purification of *n*-hexane fraction

Fractionation of the *n*-Hxn fraction (17.34 g) was conducted via column chromatography (CC) (5 x 30 cm) using a gradient solvent system of *n*-Hxn/EtOAc (0-80% EtOAc) to yield twenty-five (25) fractions. The collected fractions were combined according to their TLC profiles to obtain eleven (11) major fractions labelled in numerical order H1-H11. Subsequently, an open glass CC (2 x 40 cm) purification of H6 fraction (402.20 mg) was carried out using the gradient solvent system *n*-Hxn/EtOAc (0-50% EtOAc), resulting eleven (11) sub-fractions labeled in alphabetical order as H6A-H6K. Further purification of H6G (44.5 mg) using open CC (1 x 40 cm) with solvent system DCM/EtOAc (0-35% EtOAc), producing four (4) sub-fractions labeled in numerical order as H6G1-H6G4. Finally, the identification of compounds in sub-fraction H6G2 was carried out using LC-MS analysis.

Liquid chromatography-mass spectrometry of active sub-fraction H6G2

The sub-fraction H6G2 from the *n*-Hxn fraction of *G. thoracica* propolis was subjected to LC-MS analysis. 10 µL of sub-fraction H6G2 (1000 ppm) was auto-injected into the LC-MS, which encompassed solvent system A: water and 0.1% formic acid, B: acetonitrile and 0.1% formic acid eluted by linear gradient elution as follows: 1% B – 35% B (16 min), 35% B – 100% B (18 min) and 100% B – 1% B (20 min) at a flow rate of 0.5 mL/min. The mass spectrometer worked with high resolution electrospray ion source (HRESI) in positive mode with analyser in sensitivity mode under selected conditions with a capillary voltage of 2.50 kV, source temperature of 120 ºC, and desolvation temperature of 550 ºC. The gas flow was 50 L/h and desolvation gas was 800 L/h. The data acquisition was carried out using Waters UNIFI Scientific Information System. Molecular structures of possible compounds were drawn using ChemDraw Ultra 12.0.

2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging method

DPPH free radical scavenging activity was evaluated as described by Jo et al. [20] with some modifications. Approximately 25 µL of DMSO was added into all wells except for well A. Next, 25 µL of sample solution was added into well A and well B. Serial dilution was performed from well B until well G to produce the final concentration of 500, 250, 125, 62.5, 31.125, 15.625, and 7.183 µg/mL (*n*-Hxn fraction, H1-H11 sub-fractions) and the final concentration of 100, 50, 25, 12.5, 6.25, 3.125, and 1.563 µg/mL (H6G2 sub-fraction), respectively. 25 µL of mixture was discarded from well G. Then, 25 µL of DMSO and 200 µL of 1 mM DPPH in methanol solution were added into all wells. Subsequently, the 96 well plate was incubated in a dark condition for 30 minutes to complete the reaction. After incubation period, the absorbance was measured at 517 nm using a spectrophotometer. The blank sample consisted of DMSO and DPPH in methanol solution. Quercetin was used as positive control and the assay was carried out in triplicates.

$$\text{DPPH inhibition \%} = [(\text{Abs Blank} - \text{Abs Sample})/\text{Abs Blank}] \times 100\% \quad (1)$$

Abs Blank represents the absorbance of DMSO with 1 mM of DPPH in methanol solution while Abs Sample indicates the absorbance of the propolis samples and positive control. A graph of inhibitory percentage

against concentration of fractions was plotted to calculate the half-maximal inhibitory concentration (IC_{50}) value. The IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radicals. This could be interpolated from the dose-response graph.

2,2'-azinobis (3- ethylbenzthiazolin-6-sulfonic acid) (ABTS) free radical scavenging method

ABTS free radical scavenging activity was determined using Thaipong et al. [21] with some modifications. Approximately, 21 µL of DMSO was added into all wells except for well A. 21 µL of sample solution was added into wells A and well B. Serial dilution was performed in well B until well G to produce the final concentration of 500, 250, 125, 62.5, 31.125, 15.625, and 7.183 µg/mL (*n*-Hxn fraction, H1-H11 sub-fractions) and the final concentration of 100, 50, 25, 12.5, 6.25, 3.125, and 1.563 µg/mL (H6G2 sub-fraction), respectively. Then, 21 µL of mixture was discarded from well G and 21 µL of DMSO was added into all wells. The ABTS radical solution was diluted with methanol to obtain the absorbance value of 0.700 ± 0.200 at 734 nm. Accurately 189 µL of 7 mM ABTS radical solution was added into all well and mixed thoroughly. After 6 minutes of incubation period, the absorbance was measured at 734 nm using a spectrophotometer. The blank sample consisted of DMSO and ABTS radical solution. Additionally, Trolox was used as positive control, and the assay was carried out in triplicates.

$$\text{ABTS inhibition \%} = [(\text{Abs Blank} - \text{Abs Sample})/\text{Abs Blank}] \times 100\% \quad (2)$$

Abs Blank represents the absorbance of DMSO with ABTS radical solution while Abs Sample indicates the absorbance of the propolis samples and positive control. A graph of inhibitory percentage against concentration of fractions was plotted to calculate the IC_{50} value. The IC_{50} value is the concentration of sample required to scavenge 50% of ABTS free radicals. This could be interpolated from the dose-response graph.

Statistical analysis

All experiments were conducted in triplicate and the data was expressed as means ± standard deviation (SD). The IC_{50} values indicated the concentration of the samples that caused 50% inhibition.

Results and Discussion

Bioassay-guided fractionation for antioxidant activity

This study investigated the antioxidant activities of purified fractions of *G. thoracica* propolis using the bioassay-guided fractionation approach. The tests for antioxidant activity were carried out throughout the fractionation steps to identify the active sub-fractions.

Table 1 showed evaluation of antioxidant properties via DPPH and ABTS radical scavenging assays of *n*-Hxn fraction and sub-fractions. Sub-fraction H6 exerted the strongest DPPH antioxidant activity with IC_{50} 5.83 µg/mL while sub-fraction H2 had the weakest antioxidant activity with IC_{50} 468.60 µg/mL. Sub-fraction H1 was inactive as the IC_{50} values could not be calculated (IC_{50} >500 µg/mL). Furthermore, sub-fractions H4 and H6 showed greater antioxidant potential for ABTS assay with the IC_{50} values of 4.00 µg/mL. In contrast, sub-fraction H1 had the weakest antioxidant effect with IC_{50} 248.30 µg/mL. It is worth noting that the IC_{50} values of sub-fractions H4 and H6 are similar with Trolox, indicating stronger antioxidant capability. The highest antioxidant activity for sub-fraction H6 in the DPPH and ABTS methods can be accounted to the high amount of phenolics and flavonoids as compared to other sub-fractions. This correlates with the presence of the flavonoids, phenolics and anthocyanin compounds in sub-fraction H6G2 that were identified using the LCMS analysis. Sub-fraction H6 was further fractionated and the collected sub-fraction H6G2 was tested for its antioxidant activity. Table 2 showed the IC_{50} values for sub-fraction H6G2 in the DPPH and ABTS assays were 14.07 and 7.30 µg/mL, respectively. It indicated that this sub-fraction demonstrated highly promising antioxidant activities as evidenced by the lowest IC_{50} values.

Table 1. DPPH and ABTS radical scavenging activity of crude extract and fractions of *G. thoracica* propolis

Samples	IC_{50} Values (µg/mL)	
	DPPH	ABTS
H1	>500	248.30 ± 7.65
H2	468.60 ± 5.72	98.30 ± 4.20
H3	9.33 ± 1.16	6.50 ± 0.87
H4	6.17 ± 1.23	4.00 ± 0.00
H5	8.50 ± 1.80	4.50 ± 0.00
H6	5.83 ± 1.44	4.00 ± 0.00
H7	6.17 ± 0.58	4.83 ± 0.29
H8	11.83 ± 4.54	6.50 ± 0.87
H9	21.83 ± 1.61	6.17 ± 0.76
H10	8.17 ± 1.76	6.00 ± 1.32
H11	8.00 ± 0.87	5.00 ± 0.50
n-Hexane fraction	8.00 ± 0.05	4.00 ± 0.01
Methanol crude	9.00 ± 0.02	4.00 ± 0.01
Quercetin	4.00 ± 0.00	-
Trolox	-	4.00 ± 0.00

Data represented as means ± SD (n=3). Quercetin and Trolox serve as positive control

Table 2. DPPH and ABTS radical scavenging activity of sub-fraction H6G2 of *G. thoracica* propolis

Samples	IC_{50} values (µg/mL)	
	DPPH	ABTS
H6G2	14.07 ± 0.12	7.30 ± 2.23
Quercetin	1.67 ± 0.29	-
Trolox	-	1.70 ± 0.44

Data represented as means ± SD (n=3). Quercetin and Trolox serve as positive control

Identification of flavonoid compounds in sub-fraction H6G2 via LCMS analysis

Sub-fraction H6G2 was subjected to LCMS analysis for further identification of its antioxidant compounds and the results are presented in Table 3 and Figure 1. It contained total number of seven flavonoid, phenolic and anthocyanin compounds. The sub-fraction H6G2 contained compounds with molecular ion peaks at *m/z* 273.0761 $[M+H]^+$ and *m/z* 285.0397 $[M+H]^+$ with molecular formula of $C_{15}H_{12}O_5$ and $C_{16}H_{12}O_5$, respectively. These two compounds correspond to naringenin (**1**) and acacetin (**2**), which were reported in Turkish and Portuguese propolis, respectively [22, 23]. Furthermore, sub-fraction H6G2 also contained one compound with molecular ion peaks at *m/z* 289.0905 $[M+H]^+$ and a molecular formula of $C_{15}H_{12}O_6$. This compound corresponds to eriodictyol (**3**), which was identified by HPLC-PDA-ESI/MS in Greek propolis [24].

The molecular ion peaks at *m/z* 319.1021 $[M+H]^+$ with a molecular formula of $C_{15}H_{10}O_8$ were also detected in this sub-fraction. Such compound belongs to myricetin (**4**), which was reported in Greek and Chinese propolis [24,25]. In addition to this compound, sub-fraction H6G2 also contained a compound with molecular ion peaks at *m/z* 355.0639 $[M+H]^+$ and a molecular formula of $C_{16}H_{18}O_9$ which corresponds to chlorogenic acid (**5**). A study by Ozdal et al. [22] also reported the presence of the chlorogenic acid in Turkish propolis. Other identified compounds in this sub-fraction were eriodictyol-7-O-glucoside (**6**) and cyanidin chloride (**7**) which correspond to flavonoid glycoside and anthocyanin classes, respectively.

Sub-fraction H6G2 revealed an abundance of flavonoids whereby the LCMS data corresponded to the compounds in Figure 1. It indicated that the compounds are responsible for the antioxidant activity as reported in previous findings [22-25, 26-28].

Table 3. Q-TOF LCMS data from sub-fraction H6G2 of *G. thoracica* propolis

Compounds	Molecular Formula	Retention Time (min:sec)	*M/Z* Ratio	Ion (Adduct Ion)	Mass	Groups
Naringenin (**1**)	$C_{15}H_{12}O_5$	7.57	273.0761	$(M+H)^+$	272.0685	Flavanone
Acacetin (**2**)	$C_{16}H_{12}O_5$	17.54	285.0397	$(M+H)^+$	284.0684	Flavonoid
Eriodictyol (**3**)	$C_{15}H_{12}O_6$	14.39	289.0905	$(M+H)^+$	288.0634	Flavanone
Myricetin (**4**)	$C_{15}H_{10}O_8$	8.78	319.1021	$(M+H)^+$	318.0376	Flavonoid
Chlorogenic acid (**5**)	$C_{16}H_{18}O_9$	18.28	355.0639	$(M+H)^+$	354.0951	Phenol
Eriodictyol-7-O-glucoside (**6**)	$C_{21}H_{22}O_{11}$	11.63	450.1941	$(M)^+$	450.1162	Flavanone glycoside
Cyanidin chloride (**7**)	$C_{15}H_{11}O_6$	17.54	287.0528	$(M)^+$	287.055	Anthocyanins

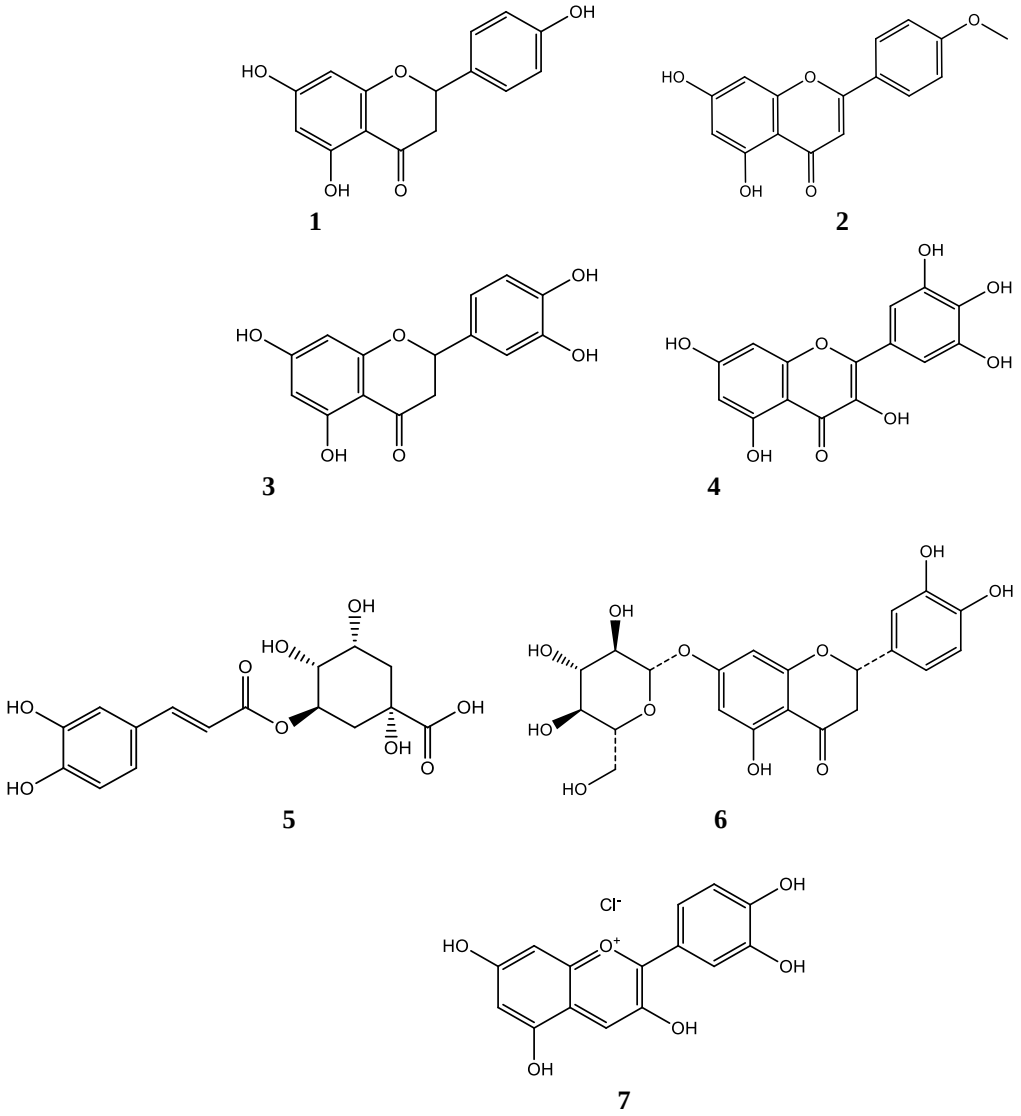

Figure 1. The structure of possible compounds identified from sub-fraction H6G2 of *G. thoracica* propolis; naringenin (**1**), acacetin (**2**), eriodictyol (**3**), myricetin (**4**), chlorogenic acid (**5**), eriodictyol-7-O-glucoside (**6**), and cyanidin chloride (**7**)

Conclusion

This study stands as a pioneer in investigating the bioassay-guided fractionation of antioxidant activity in *G. thoracica* propolis. The results conclude that *G. thoracica* propolis possesses a high potential for antioxidant activity. Subsequent fractionation of *n*-hexane fraction revealed seven flavonoid, phenolic and anthocyanin compounds that were identified by the Q-TOF LCMS analysis, namely naringenin (**1**), acacetin (**2**), eriodictyol (**3**), myricetin (**4**), chlorogenic acid (**5**), eriodictyol-7-O-glucoside (**6**), and cyanidin chloride (**7**). The antioxidant activity of *G. thoracica* propolis can be attributed to the presence of these identified compounds. It suggests that *G. thoracica* propolis has valuable source of bioactive compounds that can be applied in the food and nutraceutical products. Further investigation on the stability of propolis should be conducted to ensure their efficacy and reliability as antioxidant agents. Moreover, the mechanism underlying the antioxidant effects of these identified compounds should be further investigated to understand their contribution to the overall antioxidant capacity of propolis.

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