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Original Article

Identification of Phytochemicals and Antibacterial Activity of South Sulawesi Propolis (*Apis mellifera*) Using TLC and LC-MS/MS-QTOF

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ABSTRACT

Introduction: Propolis from *Apis mellifera* exhibits antimicrobial and antioxidant activities. Despite Indonesia's rich biodiversity, the phytochemical profile of propolis from South Sulawesi and its antibacterial potential against *Pseudomonas aeruginosa* remain underexplored. **Objective:** This study aimed to tentatively identify flavonoid and glycoside compounds in *A. mellifera* propolis from South Sulawesi and to evaluate its antibacterial activity. **Methods:** Propolis samples were collected from Masamba Subdistrict, South Sulawesi, and extracted using 70% ethanol through ultrasonic-assisted extraction. Phytochemical profiling and tentative compound identification were performed using Thin Layer Chromatography (TLC) and LC-MS/MS-QTOF. Antibacterial activity was tested by the disk diffusion method at concentrations of 0.25%, 0.5%, and 0.75%, with ciprofloxacin and distilled water as positive and negative controls. **Results:** The analysis revealed that flavonoids and glycosides were the main constituents of the extract. These compounds were tentatively identified along with other secondary metabolites, including alkaloids, terpenoids, and phenolic compounds, indicating a complex chemical composition. Antibacterial testing showed mean inhibition zones of 11.23 mm (0.25%), 13.65 mm (0.5%), and 16.50 mm (0.75%), whereas ciprofloxacin and the negative control produced zones of 31.95 mm and 5.70 mm, respectively. ANOVA revealed significant differences among treatments ($F = 232.4$, $p < 0.0001$), with the 0.75% extract showing the largest mean inhibition zone**. Conclusions:** These findings suggest that South Sulawesi propolis contains tentatively identified bioactive flavonoids and glycosides and demonstrates antibacterial activity against *Pseudomonas aeruginosa*, indicating its potential as a natural antibacterial source for further investigation.

KEYWORD: propolis (*Apis mellifera),* flavonoids, glycosides, TLC, LC-MS/MS-QTOF, antibacterial activity

INTRODUCTION

Indonesia is known as one of the world's megabiodiversity countries, hosting more than 30,000 plant species, of which approximately 9,600 have potential as medicinal plants[1,2]. This biodiversity is not only found in plants but also in animals such as honeybees (*Apis mellifera*), which produce natural substances used as raw materials in the pharmaceutical industry and scientific research. Propolis is a resinous substance mainly derived from plant resins that honeybees collect from buds and plant exudates, then mix with beeswax and salivary enzymes[3]. Flavonoids and glycosides are the main groups of secondary metabolites commonly found in plants, including roots, leaves, stems, and flowers. Flavonoids have an important role in pigmentation and aroma, and are known to exhibit various biological activities such as antibacterial, antioxidant, and anti-inflammatory properties[4].

Chemically, flavonoids are phenolic compounds with a basic 2-phenylbenzopyrone (2-phenylchromen-4-one) skeleton synthesized through the phenylpropanoid pathway. Glycosides consist of a sugar moiety (glycone) and a non-sugar moiety (aglycone) linked by a glycosidic bond, most commonly an O-glycosidic bond[5]. Both classes of compounds are abundant in propolis and contribute significantly to its pharmacological activity[6]. Propolis contains more than 200 bioactive compounds, mainly flavonoids and polyphenols, which contribute to its antibacterial mechanisms by damaging cell membranes, inhibiting protein synthesis, and enhancing the expression of natural antimicrobial peptides[7].

In addition, propolis can suppress the NF-κB signaling pathway and reduce the production of pro-inflammatory cytokines, thereby providing protective effects on body tissues[8]. The chemical composition of propolis is highly complex and depends on the floral sources as well as the geographical and climatic conditions where bees collect resins[9]. This variation results in propolis from different regions having distinct chemical profiles and biological potentials. Although several studies on propolis have been conducted in various regions of Indonesia, data regarding the chemical composition and antibacterial activity of *A. mellifera* propolis from South Sulawesi, particularly from Masamba Subdistrict, remain limited. This region, however, possesses endemic flora with the potential to produce propolis with unique characteristics. Considering the increasing resistance of *Pseudomonas aeruginosa* to various antibiotics, exploring local propolis as a natural source of antibacterial agents is of great importance. Therefore, this study aims to evaluate the antibacterial activity of propolis against *P. aeruginosa* and to tentatively identify its flavonoid and glycoside constituents using TLC and LC-MS/MS-QTOF methods.

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MATERIAL AND METHODS

Sample collection

Propolis (*Apis mellifera*) was collected from bee farms in Masamba Subdistrict, South Sulawesi, Indonesia. Samples were obtained by scraping the hive frames, then stored at -20 °C and processed into powder form.

Extraction of propolis sample

300 grams of propolis powder were extracted with 70% ethanol (1:10 w/v) using the Ultrasonic-Assisted Extraction (UAE) method for 20 minutes at 20 °C. The solution was filtered through Whatman™ filter paper, and the residue was re-extracted twice using the same procedure. The combined extract was evaporated using a rotary evaporator to obtain a concentrated propolis extract.

Thin Layer Chromatography (TLC)

The propolis extracts were analyzed using silica gel GF254 plates (7 × 2 cm; Merck®), which were activated at 100 °C for 30 minutes. Samples were spotted 1 cm above the baseline, and the solvent front was set 0.5 cm from the top edge. The mobile phase used was n-hexane:ethyl acetate (3:1) with 10% acetic acid. After elution, the plates were dried and observed under UV light (254/366 nm). Visualization reagents, including $AlCl_3$, Citroboric acid, $FeCl_3$, Dragendorff, Vanillin-sulfuric acid, and H_2SO_4, were sprayed to produce specific colors or fluorescence for tentative compound identification.

LC-MS/MS-QTOF

Preparation of standard solutions of biotin and chloramphenicol was carried out for instrument calibration and system verification. Authentic reference standards were not used for compound confirmation beyond system calibration; biotin and chloramphenicol were employed solely for instrument calibration and system performance verification. Propolis extract samples were weighed into 10 mL volumetric flasks, dissolved in methanol, and filtered through a GHP/PTFE membrane filter to remove large particles before injection into the LC-MS/MS-QTof system (Xevo G2-S QTof, Waters, USA). The analysis was performed using an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm; Waters) maintained at 40 °C, with gradient elution using mobile phase A (0.1% formic acid in ultrapure water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.6 mL/min. The mass spectrometer was operated in Time-of-Flight MS^E mode with both positive and negative electrospray ionization (ESI) to detect polar and non-polar compounds. Data were processed using the UNIFI Scientific Information System (Waters, USA) equipped with a Natural Product Library. Compound identification was performed tentatively based on agreement of m/z values, fragmentation patterns, and spectral matching with the compound library. This method refers to the Waters Application Note (Qiao et al., 2013).

Antibacterial activity (inhibition zone) test

Mueller-Hinton Agar (MHA) medium was prepared by dissolving 4.75 g of MHA in 125 mL of distilled water, sterilized using an autoclave at 121 °C for 20 minutes, then poured into sterile petri dishes and allowed to solidify at room temperature before use. The antibacterial activity test was carried out against *Pseudomonas aeruginosa* (ATCC 9721™) using the Kirby-Bauer disk diffusion method. A bacterial suspension was prepared by serial dilution with 0.9% NaCl solution until turbidity was equivalent to the 0.5 McFarland standard. Paper disks were impregnated with 20 µL of propolis extract at concentrations of 0.25%, 0.5%, and 0.75%, and placed on the surface of MHA previously inoculated with the test bacteria. Ciprofloxacin (30µg/disc) was used as a positive control, and distilled water as a negative control. All plates were incubated at 37 °C for 24 hours, and the inhibition zone diameters were measured in millimeters (mm). Data were analyzed for variance homogeneity using the Brown-Forsythe test, followed by One-Way ANOVA and Tukey's post hoc test for multiple comparisons ($p < 0.05$), with a 95% confidence level, using GraphPad Prism version 10.

RESULTS

Thin Layer Chromatography (TLC)

Figure 1. TLC analysis of the propolis extract using n-hexane:ethyl acetate (3:1) as the eluent. Spots were visualized under UV light at 254 and 366 nm, showing blue-green spots at 366 nm. After spraying with $AlCl_3$ reagent, additional blue, yellowish-green, and yellow spots were observed, while direct observation revealed yellow spots. These results indicate the tentative presence of flavonoid compounds.

Table 1 shows the range of Rf values of the observed spots, which varied from 0.05 to 0.75. Compounds with lower Rf values are generally more polar, whereas those with higher Rf values tend to be more nonpolar.

The results of TLC identification using a 10% acetic acid mobile phase showed clear separation of spots, which were visible under UV light at 254 and 366 nm. After spraying with various detection reagents, several groups of secondary metabolites, including flavonoids, phenolics, terpenoids, and alkaloids, were tentatively identified, likely present in both aglycone and glycoside forms, as shown in Figure 2. Table 2 shows the Rf values of the observed spots, which ranged from 0.03 to 0.83

LC-MS/MS-QTOF

The analysis was performed using an LC-MS/MS-QTOF instrument in ToF MS^E mode, which is part of data-independent acquisition (DIA). This mode simultaneously generates two spectra: low-energy (MS^1) for detecting intact molecular ions, and high-energy (MS^2) for obtaining fragmentation spectra, allowing the simultaneous acquisition of MS/MS data for compound identification. Various compounds were tentatively identified in both positive (ESI^+) and negative (ESI^-) ionization modes, each with a specific retention time (Rt) and mass-to-charge ratio (m/z), reflecting different polarities and molecular structures. Ion fragmentation in each mass spectrum was used to support the tentative identification of these compounds, indicating that the propolis extract contains a variety of components with diverse chemical characteristics, as shown in (Figures 3-10).

Based on LC-MS/MS-QTOF analysis, various compounds, including flavonoid aglycones and glycosides, were tentatively identified in the propolis extract. Identification was performed by comparing m/z values, retention times (Rt), fragmentation patterns, and isotope matching using the UNIFI database, which contains a mass spectral library for both positive (ESI^+) and negative (ESI^-) ionization modes. Compounds were considered tentatively identified if the mass error was ≤ 5 ppm, isotope match was ≤ 6 ppm, RMS was ≤ 10%, intensity was ≥ 300, and at least one fragment ion was present for confirmation (Tables 3 and 4).

In addition to flavonoid compounds, LC-MS/MS-QTOF analysis tentatively identified various secondary metabolites, including terpenoid aglycones and glycosides, phenolic aglycones and glycosides, as well as alkaloid aglycones. These results suggest that the propolis extract contains a variety of bioactive compounds, representing several classes of secondary metabolites with complex chemical compositions (Tables 5-9).

Antibacterial activity (inhibition zone) test

The inhibition zone results (Table 10) showed that the negative control exhibited the smallest diameter (5.70 mm), whereas the positive control (ciprofloxacin) produced the largest diameter (31.95 mm).

The propolis extract demonstrated an increase in inhibition zone with increasing concentration, with mean diameters of 11.23 mm (0.25%), 13.65 mm (0.5%), and 16.50 mm (0.75%). The Brown-Forsythe test (Table 11) indicated homogeneous variances ($p = 0.7028$). One-way ANOVA (Table 12) revealed a significant difference among treatment groups ($F(4,10) = 232.4$; $p < 0.0001$). However, Tukey's post hoc test (Table 13) showed that the differences between adjacent concentrations (0.25–0.5% and 0.5–0.75%) were not statistically significant, while both 0.5% and 0.75% differed significantly from the negative control ($p < 0.05$).

DISCUSSION

Thin Layer Chromatography (TLC)

Figure 1. Shows the results of $AlCl_3$ detection for flavonoids, which is commonly used due to its ability to form stable chelate complexes with hydroxyl (-OH) and carbonyl (C=O) groups within the flavonoid structure, particularly at the C-3, C-5, and C-4' positions. This complexation induces a bathochromic shift and enhances fluorescence,

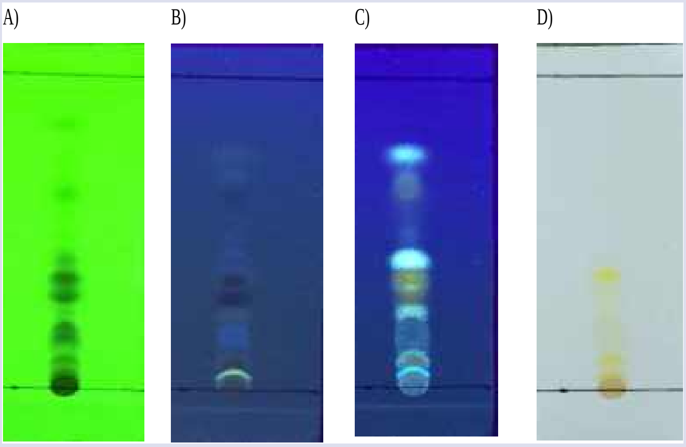

Figure 1 . Thin layer chromatography (TLC) profile of *Apis mellifera* propolis extract using n-hexane: ethyl acetate (3:1) as the mobile phase. (A) UV 254 nm, (B) UV 366 nm, (C) $AlCl_3$, (D) TLC result after spraying with $AlCl_3$ reagent.

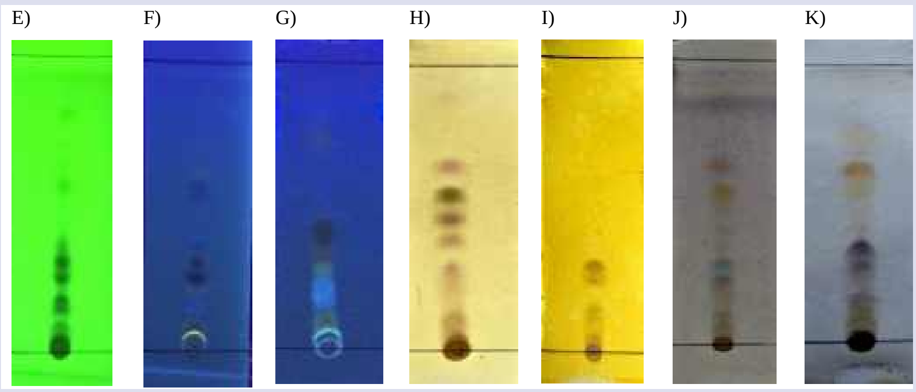

Figure 2 . Thin layer chromatography (TLC) profile of *Apis mellifera* propolis extract using 10% acetic acid as the mobile phase,(E) UV 254 nm, (F) UV 366 nm, (G) Citroboric acid, (H) $FeCl_3$, (I) Dragendorff, (J) Vanillin-sulfuric acid, (K) H_2SO_4.

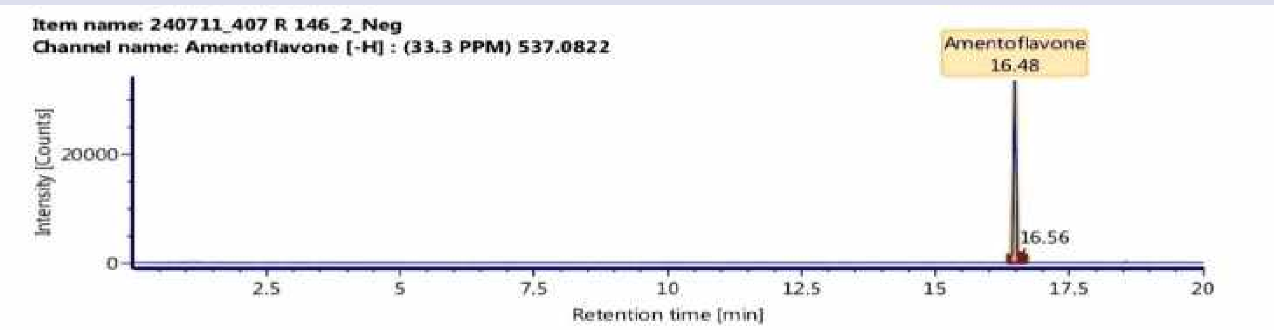

Figure 3. Tentative identification of Amentoflavone compound in Negative ESI Mode, showing a peak at 16.48 minutes (Base Peak Intensity).

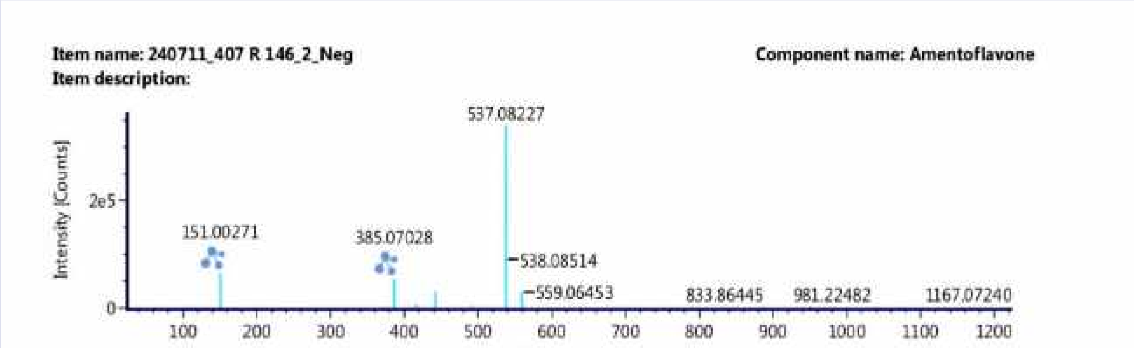

Figure 4. Tentative mass spectrum of the Amentoflavone compound, as detected in Negative ESI mode.

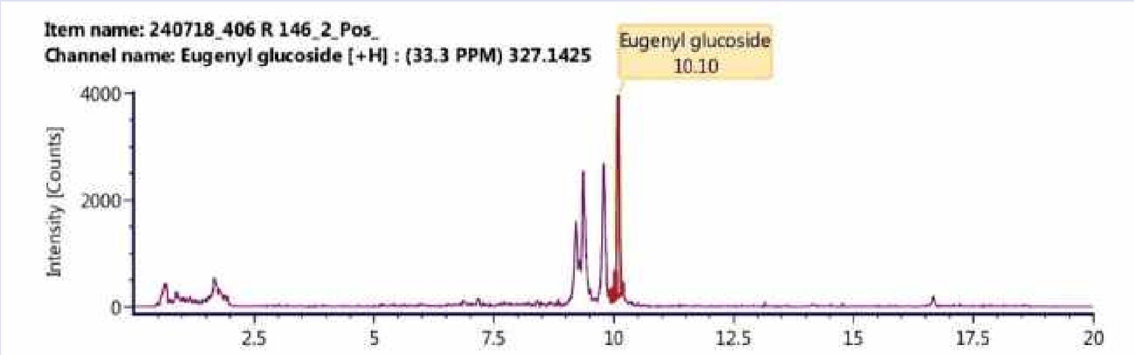

Figure 5. Tentative identification of Eugenyl glucoside compound in Positive ESI Mode, showing a peak at 10.10 minutes (Base Peak Intensity).

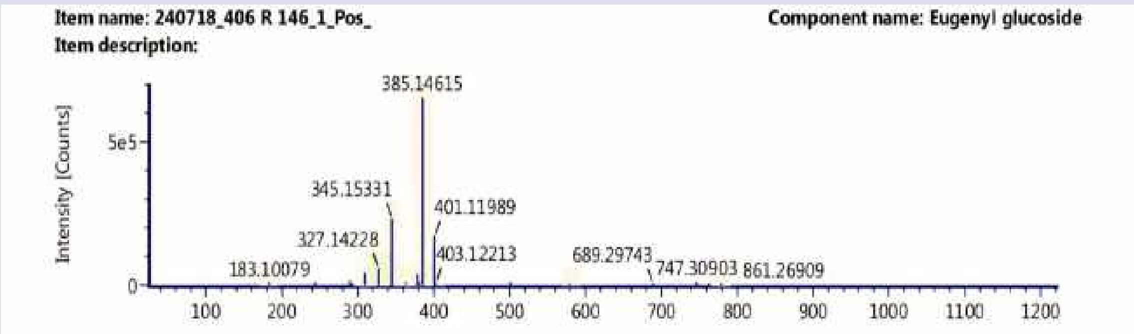

Figure 6. Tentative mass spectrum of the Eugenyl glucoside compound, as detected in Positive ESI mode.

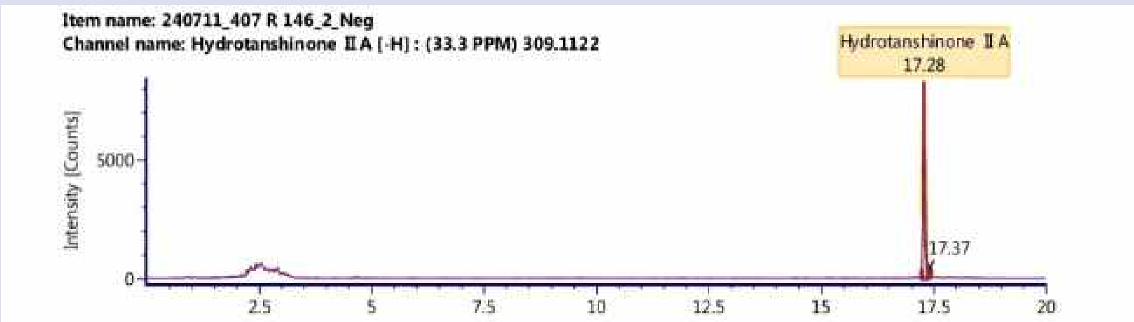

Figure 7. Tentative identification of Hydrotanshinone Ⅱ A compound in Negative ESI Mode, showing a peak at 17.28 minutes (Base Peak Intensity).

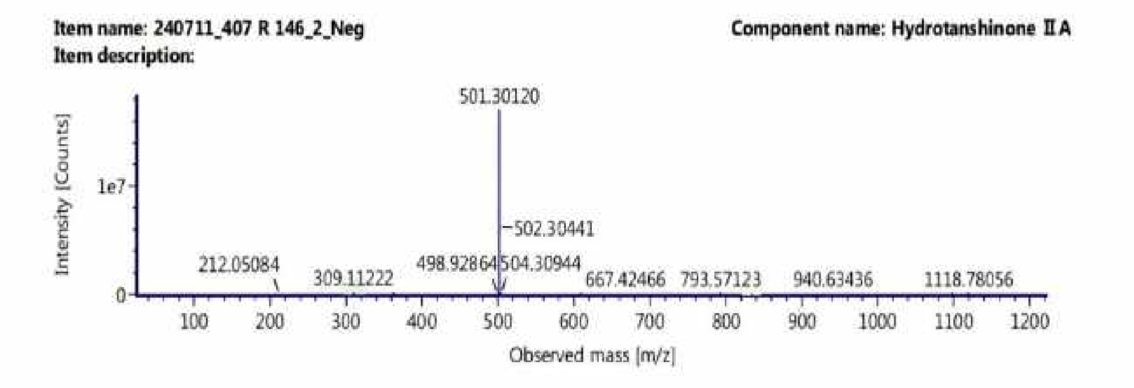

Figure 8. Tentative mass spectrum of the Hydrotanshinone Ⅱ A compound, as detected in Negative ESI mode.

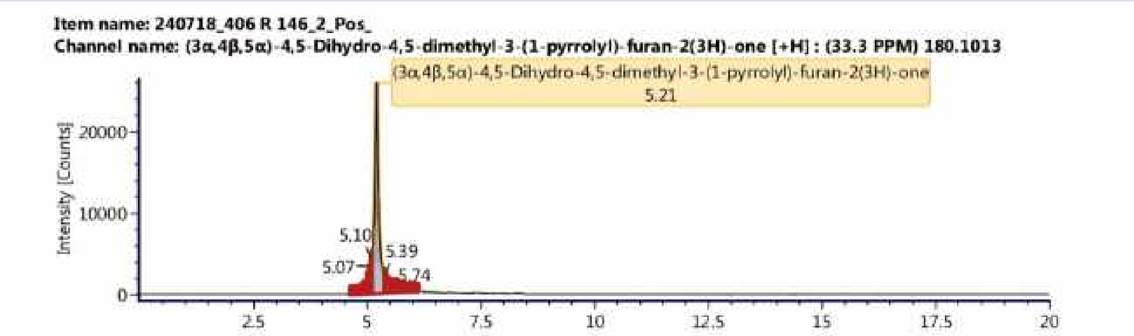

Figure 9. Tentative identification of (3α,4β,5α)-4,5-Dihydro-4,5-dimethyl-3-(1pyrrolyl)-furan-2(3H)-one compound in Positive ESI Mode, showing a peak at 5.21 minutes (Base Peak Intensity).

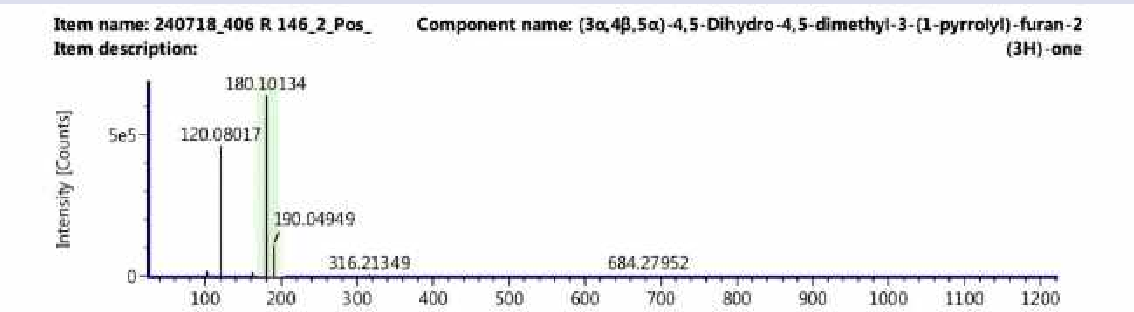

Figure 10. Tentative mass spectrum of the 3α,4β,5α)-4,5-Dihydro-4,5-dimethyl-3-(1pyrrolyl)-furan-2(3H)-one compound, as detected in Positive ESI mode.

Table 1. Rf values of propolis extract using n-hexane: ethyl acetate (3:1) as the mobile phase.

Observation type	Rf range	Description
UV λ 254nm	0.05–0.75	Visible under UV λ 254 nm
UV λ 366nm	0.05–0.62	Visible under UV λ 366nm
$AlCl_3$	0.05–0.62	Blue, yellowish-green, and yellow spots
TLC result	0.05–0.38	yellow spots

Table 2. Rf values of propolis extract using 10% acetic acid as the mobile phase.

Observation type	Rf range	Description
UV λ 254nm	0.09–0.80	Visible under UV λ 254nm
UV λ 366nm	0.09–0.80	Visible under UV λ 366nm
Citroboric	0.09–0.70	Blue, green, brown, and black spots
$FeCl_3$	0.03–0.83	Brown, purple, and brownish-green spots
Dragendorff	0.03–0.32	Brown spots
Vanillin-sulfuric acid	0.03–0.67	Green, purple, blue, yellow, and reddish-orange spots
H_2SO_4	0.05–0.72	Brown, purple, yellow, and orange spots

Table 3. Tentatively Identified Flavonoid aglycones

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Pedalitin	$C_{16}H_{12}O_7$	8.69	-2.1	3	3.05	28.55	5857	+H	317.0649
2	Mahuannin G	$C_{30}H_{22}O_{10}$	0.49	4.6	2	4.12	54.44	2769	+H	543.1311
3	Amentoflavone	$C_{30}H_{18}O_{10}$	16.48	-0.2	4	2.39	16.60	16089	-H	537.0822
4	Isoetin	$C_{15}H_{10}O_7$	11.74	-3.1	2	1.23	17.34	5070	-H	301.0343
5	Kaempferol	$C_{15}H_{10}O_6$	13.46	-2.6	3	1.55	20.24	9267	-H	285.0397

Table 4. Tentatively Identified Flavonoid glycosides

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Isorhamnetin-3-O-β-gentiobioside	$C_{28}H_{32}O_{17}$	8.69	-1.6	6	1.89	20.68	7912	+H	641.1702
2	Kaempferol-3-O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside	$C_{33}H_{40}O_{21}$	6.38	-1.6	5	1.61	12.61	3454	+H	773.2123
3	Sec-O-glucosylhamaudol	$C_{21}H_{26}O_{10}$	12.17	3.7	2	3.40	23.86	1414	+H	439.1615
4	Nepitrin	$C_{22}H_{22}O_{12}$	8.69	-0.6	5	2.10	17.23	1063	+H	479.1181
5	Isorhamnetin-3-O-β-gentiobioside	$C_{28}H_{32}O_{17}$	8.53	-0.4	5	1.26	16.60	39488	-H	639.1558
6	Isosakuranetin-7-rutinoside	$C_{28}H_{34}O_{14}$	0.85	8.8	43	11.60	32.25	4776	-H	593.1921
7	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	9.79	-1.6	1	1.44	33.29	5109	-H	593.1508
8	Kaempferol-3-O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside	$C_{33}H_{40}O_{21}$	6.21	-0.7	3	2.71	16.82	8865	-H	771.1981
9	Malvidin 3,5-diglucoside	$C_{29}H_{34}O_{17}$	9.19	-5.4	3	5.34	2.71	1235	-H	653.1774
10	Nelumboroside B	$C_{33}H_{40}O_{20}$	6.68	-0.8	1	7.39	10.59	1298	-H	755.2046
11	6-Methoxykaempferol-3-O-β-D-galactopyranoside	$C_{22}H_{22}O_{12}$	10.28	-1.5	4	1.19	5.75	1282	-H	477.1047

Table 5. Tentatively Identified Phenolic glycosides

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Eugenyl glucoside	$C_{16}H_{22}O7$	10.10	-4.2	3	5.31	17.25	2090	+H	327.1425
2	Tenuifoliside D	$C_{18}H_{24}O9$	10.10	-8.0	4	8.21	43.50	23005	+H	385.1462
3	4-O-Caffeoylquinic acid	$C_{16}H_{18}O_9$	5.78	-8.3	1	4.97	13.28	1439	-H	353.0862
4	Ephedrannin B	$C_{30}H_{20}O_{10}$	16.38	-5.5	1	6.89	19.70	1418	-H	539.0954

Table 6. **Tentatively Identified Phenolic aglycones**

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Difengpin	$C_{20}H_{20}O_4$	16.68	-1.3	2	5.18	10.21	1103	+H	325.1430
2	2H-1-Benzopyran-2-one	$C_9H_6O_2$	1.69	-7.4	3	7.47	28.65	2117	+H	147.0430
3	Isomexoticin	$C_{16}H_{20}O_6$	10.10	-0.7	1	0.80	17.26	1328	+H	309.1331
4	Pinoresinol dimethyl ether II	$C_{22}H_{26}O_6$	16.64	-2.2	3	4.03	15.29	1604	+H	387.1794
5	p-Hydroxy-phenylpropionic acid	$C_9H_{10}O_3$	7.55	-6.0	1	2.21	4.24	1954	-H	165.0541
6	Wedelolactone	$C_{16}H_{10}O_7$	15.35	-0.9	3	9.69	4824.88	1137	-H	313.0351

Table 7. **Tentatively Identified Terpenoid aglycones**

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Hydrotanshinone IIA	$C_{19}H_{18}O_4$	17.28	-3.3	7	1.49	8.15	3492	-H	309.1122
2	Miltiodiol	$C_{19}H_{22}O_3$	18.35	8.7	5	11.91	156.70	21407	-H	297.1522
3	Rapanone	$C_{19}H_{30}O_4$	18.47	8.2	2	12.02	20.89	17617	-H	321.2098
4	Bufotalinin	$C_{24}H_{30}O_6$	16.69	-2.2	1	2.08	23.59	4734	+H	415.2106
5	Decumbesterone A	$C_{29}H_{46}O_7$	16.99	-7.4	2	6.97	17.24	1108	+H	507.3279
6	Protodiosgenin	$C_{33}H_{54}O_9$	16.99	-6.3	9	7.18	23.47	2003	+H	595.3803
7	Daturametelin H	$C_{34}H_{46}O_9$	17.80	8.4	2	9.72	34.82	2295	-H	597.3089

Table 8. **Tentatively Identified Terpenoid glycosides**

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	Trillin	$C_{33}H_{52}O_8$	17.19	-2.2	16	8.53	27.75	1717	+H	577.3722
2	Timosaponin B-2	$C_{45}H_{76}O_{19}$	16.57	-8.0	179	7.34	2079.66	3169	-H	919.4946
3	Ginsenoside F3	$C_{41}H_{70}O_{13}$	16.98	-3.7	28	3.34	36.30	2098	+H	771.4861
4	Gypenoside XI	$C_{48}H_{82}O_{17}$	17.57	-3.2	15	3.19	6.40	136486	+H	931.5595
5	Fuzinoside	$C_{15}H_{28}O_{13}$	2.01	-4.8	3	5.16	27.26	959	-H	415.1426
6	Lucidumoside C	$C_{27}H_{36}O_{14}$	16.63	-9.4	16	8.08	9.65	7397	-H	583.1977

Table 9. **Tentatively Identified Alkaloid aglycones**

No.	Compound Name	Molecular Formula	Retention Time (min)	Mass Error (ppm)	Total Fragments	Isotope Match M/z RMS PPM	Isotope Match Intensity RMS percent (%)	Response	Mode ESI	Precursor Ion (m/z)
1	(3α,4β,5α)-4,5-Dihydro 4,5-dimethyl-3-(1pyrrolyl)-furan-2(3H)-one	$C_{10}H_{13}NO_2$	5.21	-3.2	1	3.55	22.70	14539	+H	180.1013
2	Psamosilenins A	$C_{51}H_{64}N_8O_8$	16.55	-6.9	97	13.55	67.71	2211	-H	915.4712

Table 10. **Inhibition Zone of Propolis Extract**

Extract Concentration (%)	Average Inhibition Zone (mm)	N	Letter Notation
0.75 %	16.50	3	b
0.5 %	13.65	3	b,c
0.25 %	11.23	3	c
Positive Control	31.95	3	a
Negative Control	5.70	3	d

Letter notations indicate significant differences based on Tukey's HSD test ($p < 0.05$). Each treatment was tested in triplicate ($n = 3$).

Table 11. Brown-Forsythe Test for Homogeneity of Variances

Test Statistic	Value
F-value (DFn, DFd)	0.5512 (4.10)
(p-value)	0.7028
Significance Interpretation	Not significant ($p > 0.05$)
Conclusion	Homogeneity of variances confirmed

Table 12. One-Way ANOVA Results On Inhibition Zones

Source of variation	SS	df	MS	F (4,10)	p-value
Between Treatments	1166	4	291.6	232.4	<0.0001
Within Treatments (Error)	12.55	10	1.255	-	-
Total	1179	14	-	-	-

Table 13. Tukey's HSD Test Results

Comparison	Mean Difference (mm)	95% CI	p-value	significant
0.25% vs 0.5%	-2.417	-5.427 to 0.593	0.1349	No
0.25% vs 0.75%	-5.267	-8.277 to -2.257	0.0013	Yes
0.25% vs positive control	-20.72	-23.73 to -17.71	<0.0001	Yes
0.25% vs negative control	+5.533	+2.523 to +8.543	0.0009	Yes
0.5% vs 0.75%	-2.850	-5.860 to 0.159	0.0678	No
0.5% vs positive control	-18.30	-21.31 to -15.29	<0.0001	Yes
0.5% vs negative control	+7.950	+4.940 to +10.96	<0.0001	Yes
0.75% vs positive control	-15.45	-18.46 to -12.44	<0.0001	Yes
0.75% vs negative control	+10.80	+7.790 to +13.81	<0.0001	Yes
positif vs negative control	+26.25	+23.24 to +29.26	<0.0001	Yes

producing a yellow to greenish-yellow coloration under UV light at 366 nm[10]. Furthermore, flavonoids that react with $AlCl_3$ may also exhibit yellow to bluish-yellow fluorescence when observed under UV 366 nm, which serves as a characteristic indicator of flavonoid presence[11]. As shown in Table I, the Rf values obtained from TLC using $AlCl_3$ reagent ranged from 0.05 to 0.62, indicating the presence of compounds with varying polarity, from nonpolar to polar. These findings are consistent with previous reports suggesting that flavonoids with lower Rf values (approximately 0.3-0.4) are typically more polar, such as flavonoid glycosides, while those with higher Rf values (around 0.6-0.7) tend to be less polar or nonpolar, such as aglycone flavonoids[12,13]. These TLC results support the tentative identification of flavonoid classes in the propolis extract.

In the TLC results for glycosides shown in Figure 2, several groups of secondary metabolites were detected, including phenolics, terpenoids, and alkaloids, which were identified based on their characteristic coloration on the TLC plate. Phenolic compounds generally exhibited purple coloration[14], terpenoids appeared as blue, bluish-purple, or red-purple spots, while alkaloids were characterized by yellow, yellowish-orange, or orange-brown colors[11,15]. The Rf values obtained (Table 2) showed considerable variation among the spots, indicating differences in polarity levels of the separated compounds. This variation can be attributed to the structural characteristics of glycosides, in which the sugar moiety (glycone) is bound to the non-sugar portion (aglycone) through a glycosidic linkage. The presence of this linkage increases the overall polarity of glycosides compared to their aglycones, causing them to migrate more slowly in the polar mobile phase and produce relatively lower Rf values. Therefore, the differences observed in Rf values can be used as a comparative parameter to assess polarity levels and to support the tentative identification of glycosidic compounds within the extract[12].

LC-MS/MS-QTOF

Based on LC-MS/MS-QTOF analysis, the propolis extract exhibited a complex metabolite profile. The results were presented as a Base Peak Intensity (BPI) chromatogram, showing the main peaks according to retention times (Rt). MS/MS fragmentation data provided supportive evidence for the tentative identification of several compounds, such as amentoflavone and eugenyl-β-D-glucoside, based on their characteristic ion patterns. Detailed interpretation of individual fragmentation pathways was not performed, as this study focused on metabolite profiling and tentative identification of compounds present in the propolis extract.

A total of 41 compounds were tentatively identified and classified into flavonoids, phenolics, terpenoids, and alkaloids. These compounds were detected in both glycosidic and aglycone forms, reflecting the chemical diversity of the bioactive constituents present in the propolis extract. Within the flavonoid group, compounds such as pedalitin, nelumboroside B, amentoflavone, and nepitrin were tentatively identified in either aglycone or glycosidic forms. Flavonoids represent one of the major classes of secondary metabolites in propolis and are widely reported to exhibit antioxidant and antibacterial activities. Amentoflavone, a natural biflavonoid that has been reported in plants such as *Selaginella tamariscina* and in propolis, was tentatively identified in this study. This compound is widely reported to exhibit antibacterial, antiviral, antioxidant, and anti-inflammatory activities. Its antibacterial activity has been demonstrated against *Staphylococcus aureus* and *Escherichia coli* through mechanisms involving disruption of bacterial cell membranes and inhibition of key metabolic enzymes. The hydroxyl groups in its structure contribute to free radical scavenging, underpinning its antioxidant effect, while suppression of COX-2, iNOS, and NF-κB expression accounts for its anti-inflammatory activity[16]. Moreover, amentoflavone has been shown to enhance the efficacy of antibiotics such as ampicillin and cefotaxime, with FIC values ≤ 0.5, through hydroxyl radical formation and NADH depletion that induce oxidative stress in bacterial cells[17].

Phenolic compounds also play an important role in antioxidant and antimicrobial activities, particularly through their ability to donate hydrogen atoms and stabilize free radicals. One tentatively identified compound, eugenyl-β-D-glucoside, can be hydrolyzed by enzymes and microorganisms in human saliva, releasing the more biologically active aglycone, eugenol[18]. This indicates that glycosidic forms in propolis may serve as precursors to active compounds, which exhibit optimal activity after enzymatic conversion. A derivative of eugenol glycoside, eugenol α-D-glucopyranoside, has been reported to have enhanced stability and specific biological activities[19]. These findings suggest that phenolic glycosides not only act as metabolite reserves but may also contribute to the biological activity of propolis, depending on the conditions and biological environment.

Additionally, LC-MS/MS-QTOF analysis tentatively identified terpenoid and alkaloid compounds. Propolis contains various bioactive compounds, including terpenoids and alkaloids, which have been widely reported to exhibit antibacterial activity. These compounds have been reported to be effective against a wide range of pathogenic bacteria, including Gram-positive, Gram-negative, periodontitis-associated pathogens, and multidrug-resistant uropathogens. The chemical composition of propolis is influenced by its geographical origin and the types of plants utilized by bees, resulting in considerable chemical variability[20,21,22].

In LC-MS/MS-based metabolite profiling, low-intensity signals may increase the risk of false-positive identification due to insufficient fragmentation information or reduced spectral quality. In the present study, all reported compounds exhibited signal intensities above the predefined acceptance threshold (≥300), thereby minimizing the risk of false-positive identification. Nevertheless, compound identification remains tentative, as it was based on accurate mass measurements, fragmentation patterns, and database matching without confirmation using authentic reference standards.

Antibacterial activity (inhibition zone) test

The results of the variance homogeneity test (Brown-Forsythe, $p = 0.7028$) shown in Table 11 indicate that the data were homogeneous, allowing further analysis using one-way ANOVA. The ANOVA results (Table 12) showed a significant difference among the treatment groups ($F(4,10) = 232.4$; $p < 0.0001$). However, the Tukey HSD post hoc test (Table 13) demonstrated that not all pairwise comparisons were statistically significant, as no significant differences were observed between the 0.25-0.5% ($p = 0.1349$) and 0.5-0.75% ($p = 0.0678$) concentrations. These findings suggest an increasing trend in antibacterial activity with higher concentrations, although differences between adjacent concentrations were not statistically significant within the tested range.

The observed increase in antibacterial activity of propolis aligns with previous studies reporting a similar concentration-dependent relationship. Several reports have shown that inhibition zone diameters increase with higher concentrations of propolis extract, although the activity against Gram-negative bacteria tends to be lower than that against Gram-positive bacteria[23]. Propolis has also been demonstrated to inhibit both the growth and biofilm formation of *Pseudomonas aeruginosa*, with greater effectiveness observed at higher concentrations[24]. Furthermore, increasing the concentration of ethanolic propolis extract has been associated with decreased MIC values and enlarged inhibition zones against *Escherichia coli* and *P. aeruginosa*, which is attributed to higher levels of flavonoids and phenolic compounds[25]. Similarly, formulations of propolis gel at medium to high concentrations produced inhibition zones of approximately 10-16 mm against *P. aeruginosa*[26], while propolis nanoemulsions achieved inhibition zones of around 17 mm at high concentrations[27]. Overall, the *Apis mellifera* propolis extract examined in this study exhibited a concentration-dependent increase in antibacterial activity, with 0.75% extract showing the largest mean inhibition zone within the tested concentration range. Although the disk diffusion assay demonstrated antibacterial activity of the propolis extract, this method does not provide quantitative parameters such as the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). Therefore, further studies determining MIC and/or MBC values are strongly recommended to strengthen antibacterial claims and enable direct comparison with existing literature. It should also be noted that the disk diffusion method has inherent limitations when applied to viscous or chemically complex extracts such as propolis, as diffusion through agar may be restricted. This limitation may influence the size of the inhibition zones and should be considered when interpreting antibacterial activity. Although its antibacterial effect was not as strong as that of standard antibiotics, these findings reinforce the potential of propolis as a natural antibacterial agent, particularly for topical or oral health applications. Moreover, the results suggest a basis for further investigation into the synergistic effects of propolis combined with conventional antibiotics for the treatment of *P. aeruginosa* infections, given this bacterium's notable resistance to multiple antimicrobial agents.

CONCLUSION

The *Apis mellifera* propolis extract from Masamba Subdistrict, South Sulawesi, Indonesia, exhibited a complex metabolite profile, with flavonoids, phenolics, terpenoids, and alkaloids tentatively identified using TLC and LC-MS/MS-QTOF. All reported compounds had signal intensities ≥300, minimizing the risk of false-positive identification, though identities remain tentative and require confirmation using authentic reference standards. Antibacterial testing against *Pseudomonas aeruginosa* showed a concentration-dependent increase in activity, with 0.75% extract showing the largest mean inhibition zone; differences between some adjacent concentrations were not statistically significant and should be interpreted cautiously. Future studies are recommended to determine MIC and/or MBC values and to perform more detailed LC-MS/MS analyses for structural confirmation and to clarify the specific roles of bioactive metabolites.

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