SUPPLEMENTARY MATERIAL

Chemical profiling and biological activity evaluation of propolis from Çayeli-Rize, Eastern Black Sea Region, Anatolia

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Abstract

The constituents of the aqueous, ethanol, hexane, and methanol extracts of Anatolian propolis collected from the Eastern Black Sea Region (Çayeli-Rize) were investigated by GC-MS, HPLC, and AAS. Interestingly, lactulose has been identified. Ten phenolic compounds, namely caffeic acid, ferulic acid, rutin, taxifolin, quercetin, kaempferol, apigenin, silicristin, silibinin, and gallic acid were determined. The contents of phenolic acids and flavonoids varied between 17.04-642.59 ppm and 1.18-2749.20 ppm, respectively. Minerals found in propolis were Na, K, Ca, Mg, and Zn. The methanol extract had the highest antiproliferative activity against the A549 cell line with an IC_{50} value of 0.1821 μL/mL. The aqueous extract showed very high selectivity for HEK-293T healthy cells. The extracts showed higher antibacterial activity against Gram-negative bacteria compared to Gram-positive bacteria.

Keywords: Propolis; chemical composition; flavonoids; antiproliferative activity; antibacterial activity

1. Experimental

1.1. Propolis origin

The raw propolis samples were obtained in 2021 from Çataldere Bal Organic Beekeeping House (TR-OT-06-3322), located in Çayeli (40°53′59″N, 40°45′48″E) in the Çataldere district of Rize province, Türkiye. The propolis provided by the company was sourced from hives of the Caucasian honeybee (*Apis mellifera caucasia*). Samples were packed in plastic bags and stored in a refrigerator (4-6ºC) until analysis. The Çayeli region, characterized by a temperate climate with abundant rainfall throughout the year and mild winters, supports a lush forest ecosystem. The dominant tree species include fir (*Abies* spp.), spruce (*Picea* spp.), Scots pine (*Pinus sylvestris*), cedar (*Cedrus* spp.), beech (*Fagus* spp.), oak (*Quercus* spp.), chestnut (*Castanea sativa*), linden (*Titia* spp.), elm (*Ulmus* spp.), hornbeam (*Carpinus* spp.), alder (*Alnus* spp.), and hazelnuts (*Corylus* spp.) (Sarac et al. 2013; Atalay 1983; Usta et al. 2018). Notable medicinal plants in the region include *Angelica sylvestris* L., *Senecio vulgaris* L., *Buxus sempervirens* L., *Silene vulgaris* (Moench) Garcke, *Glechoma hederacea* L., *Lamium album* L., *Lamium purpureum* L., Linum catharticum L., *Ligustrum vulgare* L., *Oxalis acetosella* L., *Plantago lanceolata* L., *Dactylis glomerata* L., *Primula vulgaris* Huds., *Ranunculus ficaria* L., *Sorbus aucuparia* L., *Galium odoratum* (L.) Scop., and *Salix caprea* L. (Baykal et al. 2011).

1.2. Extraction procedures

The conventional Soxhlet extraction apparatus, consisting of a condenser with running water, a Soxhlet extractor, and a round bottom flask was used for the extraction. 40 grams of dried and ground propolis were placed in a Soxhlet apparatus and subjected to 24 hours of Soxhlet extraction, using 350 mL of solvent. Four different solvents were used: ethanol, methanol, hexane, and water. The extraction temperature was set according to the boiling point of each solvent. The resulting extracts were gathered for analysis.

1.3. Characterization of the extracts

1.3.1. Gas Chromatography-Mass Spectrometry

The gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent 7890B gas chromatograph coupled to an Agilent-5977A mass selective detector under electron impact ionization (70 eV), equipped with a quadrupole analyzer and an Agilent Chem Station data system. GC separation was conducted on an HP-5ms Ultra Inert 30 m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (Agilent 19091S-433UI). Helium at a flow rate of 1.0 mL/min was used as the carrier gas. The injector and detector temperatures were set at 280°C. The injection was performed in split mode with a split ratio of 10:1 (v/v). 1 μL of the sample was injected into the capillary column. The solvent delay time was 4 minutes. The extracts were analyzed with the column initially held at 80°C for 1 minute, then

increased at a rate of 4°C/min to 300°C, and held at 300°C for 9 minutes. Mass spectra were interpreted by comparison with the peak distribution database of the National Institute of Standard and Technology (NIST MS 14.0, Gaithersburg, MD, USA). Methoxyamine hydrochloride (Merck-89803) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (Merck-841102) were used as derivatizing agents with spectrophotometric grade pyridine (Merck-7460). The extracts were freeze-dried for 48 hours. For derivatization, 20 µL of methoxyamine hydrochloride in pyridine (30 mg/mL) was added as the first derivatizing agent and incubated at 37ºC for 17 hours. Then, the second derivatizing agent, 90 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), was added and incubated at 3ºC for 2 hours before analysis by GC-MS.

1.3.2. High Performance Liquid Chromatography

Chromatographic analyses were performed using an HPLC device (Dionex, Germany). An Ascentis Express (10 cm x 3 mm) column with a particle size of 2.7 µm was maintained at 25ºC. The mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and a mixture of acetonitrile and methanol (3:1 v/v) (Solvent B). The gradient grade profile was as follows: 20% solvent B at 0 min, 40% solvent B at 5 min, 70% solvent B at 10 min, 80% solvent B at 15 min, and 80% solvent B at 20 min, with a flow rate of 0.5 mL/min. The total run time was 20 min. The injection volume was 10 µL. Referans standards including caffeic acid, ferulic acid, rutin, gallic acid, silibinin, quercetin, and apigenin were purchased from Sigma (Darmstadt, Germany) with purities ≥ 98%. Taxifolin and silychristin were obtained from Fluka (North Carolina, USA) with purities ≥ 98%, while kaempferol (purity ≥ 95%) was purchased from Cayman (Michigan, USA). Ethanol (purity ≥ 99.9%), acetonitrile (purity ≥ 99.9%), and formic acid (purity ≥ 99.9%) were used as mobile phases and/or solvents, sourced from Merck (Gernsheim, Germany).

1.3.2. Atomic Absorption Spectroscopy

The mineral composition of propolis was analyzed using a Varian AAS240Z model graphite furnace atomic absorption spectrometer (Mulgrave Virginia, Australia), equipped with a PSD-120 model autosampler, a TGA-120 model thermal graphite furnace atomizer, a Zeeman background correction system, and pyrolytically coated carbon tubes (Varian, P/N-63-100,012-00). Absorbance signals were evaluated using the Varian SpectraAA software program, employing peak area mode for absorbance measurements. The instrument settings and operating conditions were as specified in the software provided by the manufacturer. Argon gas (99.98%, Oksan, Ankara, Türkiye) was used in the heating steps of the program, except for the atomization step.

1.4. Biological assays

1.4.1. Antiproliferative activity studies

The human lung cancer cell line (A549) (ATCC® CCL-185™) was procured from the American Type Culture Collection (ATCC, USA). Cytotoxicity studies were conducted following previously published procedure (Akkoc 2019). The A549 and HEK-293T cells were seeded into sterile 96-well plates at a density of 5 x 10^3 cells/well. After 24 hours, the cells were exposed to compounds at four different concentrations for 72 hours. Following the incubation period, 5 mg/mL of MTT stock solution was added to each well, and the plates were incubated for an additional 2 hours. Absorbance values were measured using a Promega plate reader device at 590 nm. IC_{50} values were calculated using GraphPad Prism software 5.

1.4.2. Antimicrobial activity

The propolis extracts were assessed for their antimicrobial activity against pathogenic Gram-positive bacteria (*Staphylococcus epidermidis* ATCC12228, *Bacillus cereus* RSKK 863) and Gram-negative bacteria (*Salmonella typhi* NCTC901.8394, *Proteus vulgaris* RSKK 96026, *Pseudomonas aeroginosa* ATCC27853, *Enterobacter aerogenes* ATCC 51342, *Klebsiella pneumonia* ATCC 700603), as well as a yeast strain (*Candida albicans* Y-1200-NIH). Testing was conducted using the well diffusion method at various concentrations (0.1 ppm, 0.5 ppm, 1 ppm, 1.5 ppm, and 2 ppm), following the procedure outlined by Rubab et al. (2021). The pathogenic bacteria and yeast were also evaluated for their resistance to four antibiotics (Kanamycin (30 µg), Sulfamethoxazole (25 µg), Ampicillin (10 µg), and Amoxicillin (30 µg)), and one antifungal agent (Nystatin (100 µg)). The solvents used for preparing propolis extracts (water, ethanol, methanol, and hexane) were also tested as controls to assess their antimicrobial activity.

1.5. Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test using Graphpad Prism, version 8.0.2 (Graphpad Software Inc., CA, USA). All data were expressed as the mean values±SD. $P<0.05$ was considered statistically significant.

2. Figures

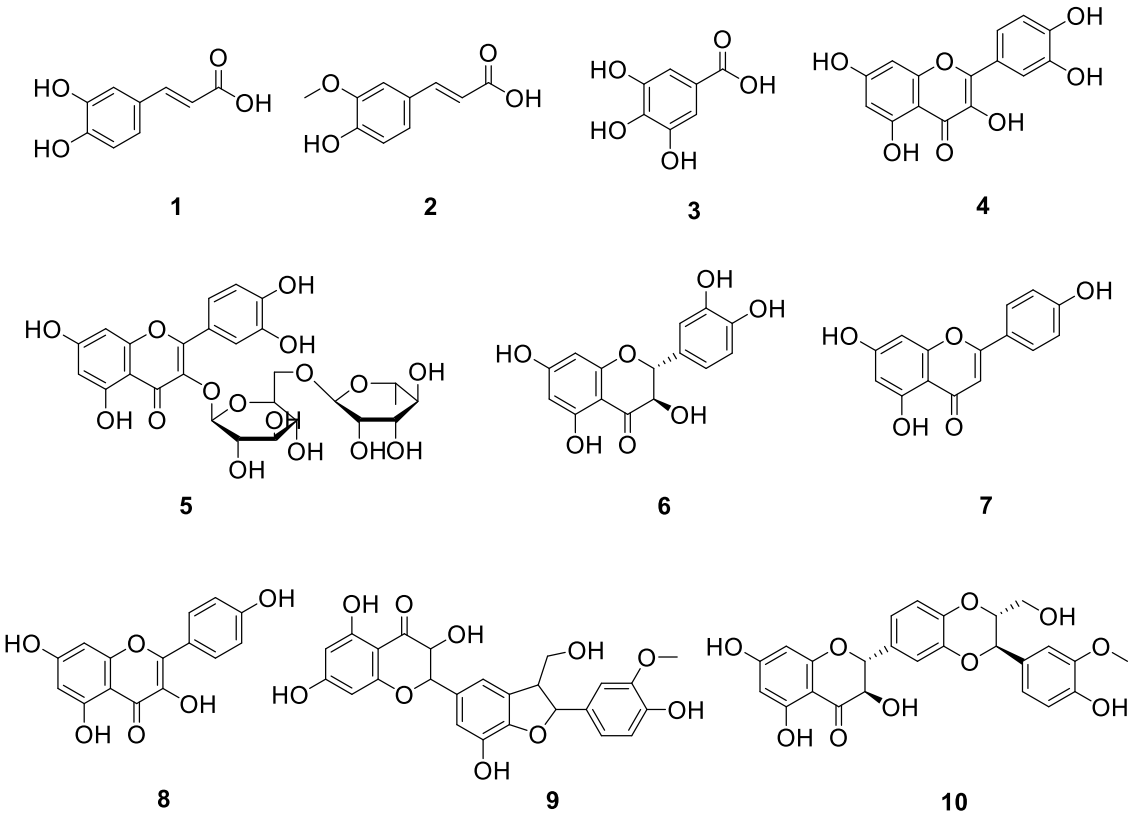
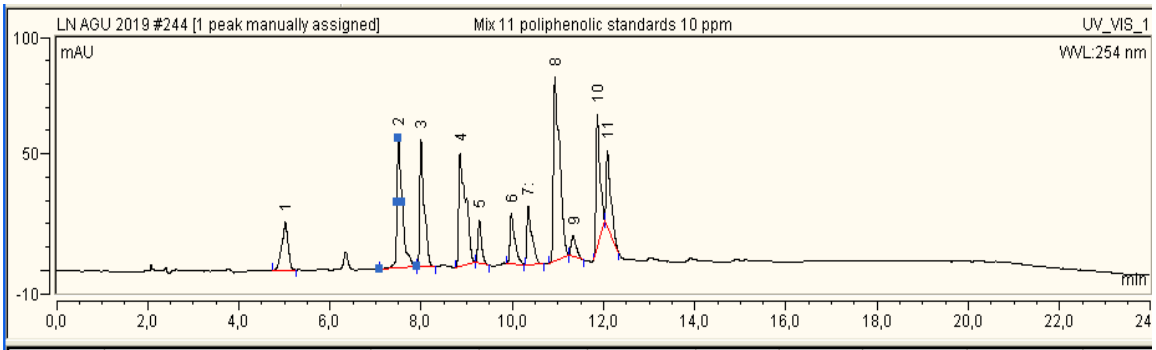
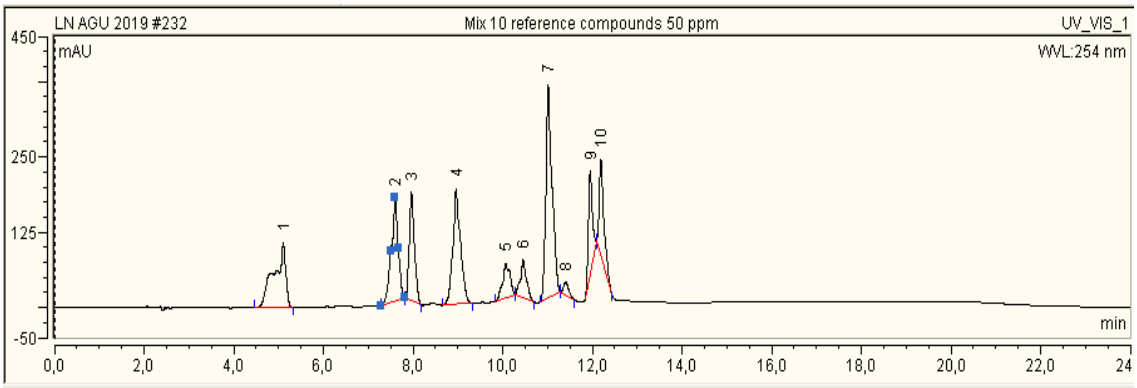


Figure S1. Structres of identified phenolic compounds: **1**. caffeic acid, **2**. ferulic acid, **3**. gallic acid, **4**. quercetin, **5**. rutin, **6**. taxifolin, **7**. apigenin, **8**. kaempferol, **9**. silichristin, **10**. silibinin.



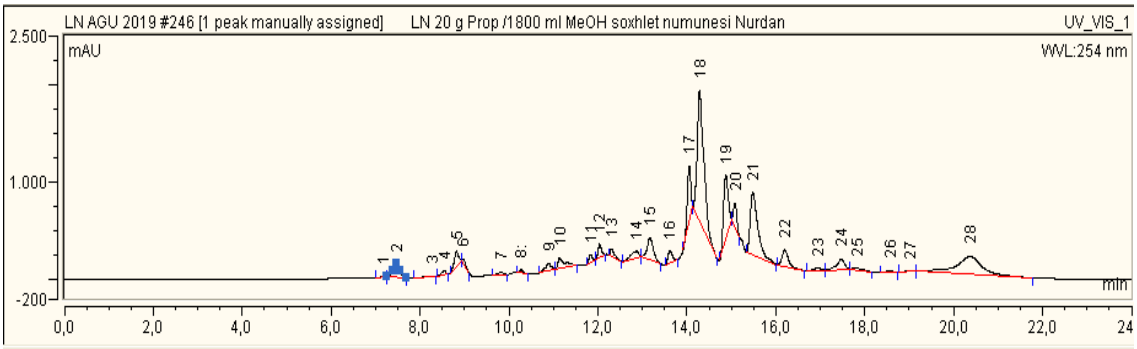
Sample No.	Sample Name	Ret.Time min [7.31 - 7.69] UV_VIS_1	Area mAU*min [7.31 - 7.69] UV_VIS_1	Height mAU [7.31 - 7.69] UV_VIS_1	Amount [7.31 - 7.69] UV_VIS_1	Type [7.31 - 7.69] UV_VIS_1	Plates (EP) [7.31 - 7.69] UV_VIS_1	Time
244	Mix 11 poliphenolic standards 10 ppr	7,502	7,5322	55,6352	n.a.	BMB	21773	02,12,21 14:09
245	LN 20 g Prop /180 ml der MeOH sox	7,489	133,7737	818,6263	n.a.	bMB	13719	02,12,21 14:34
246	LN 20 g Prop /1800 ml MeOH soxhle	7,469	22,6086	138,9084	n.a.	bMB	13115	02,12,21 15:02
247	LN 20 g Prop /2000 ml EtOH soxhlet	7,445	19,9491	74,2877	n.a.	BMB	6715	02,12,21 15:27

Figure S2. HPLC chromatogram of eleven different reference compounds at 10 ppm: **1**. gallic acid, **2**. caffeic acid, **3**. rutin, **4**. ferulic acid, **5**: taxifolin, **6**. silychristin, **7**. resveratrol, **8**. quercetin, **9**. silibinin, **10**. apigenin, **11**. kaempferol.



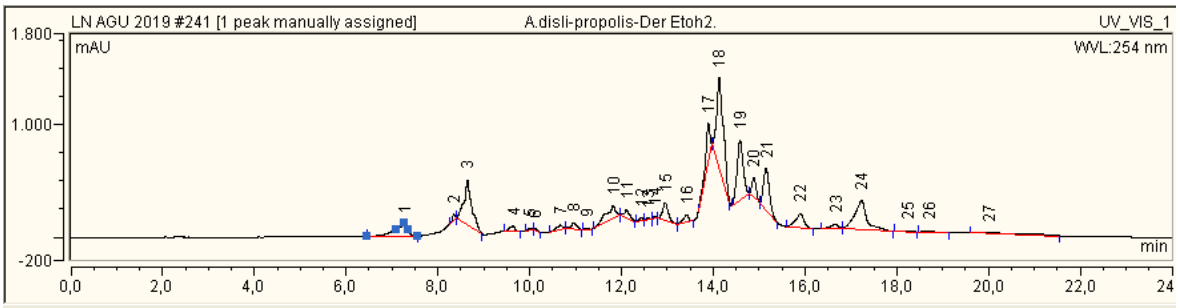
Sample No.	Sample Name	Ret.Time min [7.41 - 7.79] UV_VIS_1	Area mAU*min [7.41 - 7.79] UV_VIS_1	Height mAU [7.41 - 7.79] UV_VIS_1	Amount [7.41 - 7.79] UV_VIS_1	Type [7.41 - 7.79] UV_VIS_1	Plates (EP) [7.41 - 7.79] UV_VIS_1	Time
232	Mix 10 reference compounds 50 ppm	7,603	30,3338	171,1906	n.a.	BMb	10888	07,06,21 12:20

Figure S3. HPLC chromatogram of ten different reference compounds at 50 ppm: **1**. gallic acid, **2**. caffeic acid, **3**. rutin, **4**. ferulic acid, **5**. silychristin, **6**. resveratrol, **7**. quercetin, **8**. silibinin, **9**. apigenin, **10**. kaempferol.



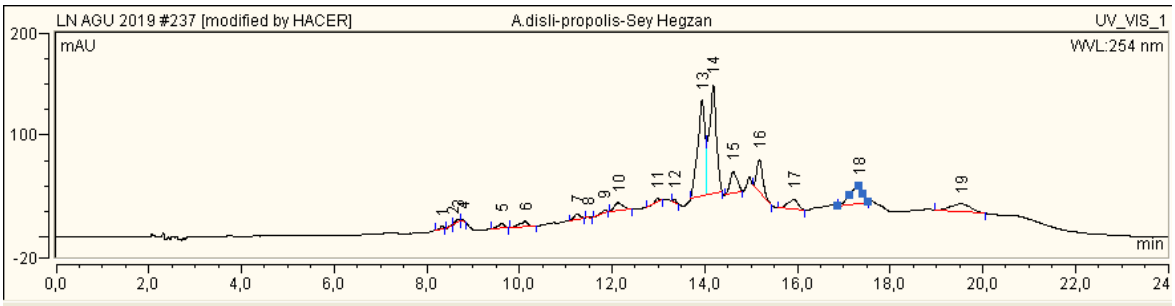
Sample No.	Sample Name	Ret.Time min [7.28 - 7.66] UV_VIS_1	Area mAU*min [7.28 - 7.66] UV_VIS_1	Height mAU [7.28 - 7.66] UV_VIS_1	Amount [7.28 - 7.66] UV_VIS_1	Type [7.28 - 7.66] UV_VIS_1	Plates (EP) [7.28 - 7.66] UV_VIS_1	Time
244	Mix 11 poliphenolic standards 10 ppr	7,502	7,5322	55,6352	n.a.	BMB	21773	02,12,21 14:09
245	LN 20 g Prop /180 ml der MeOH sox	7,489	133,7737	818,6263	n.a.	bMB	13719	02,12,21 14:34
246	LN 20 g Prop /1800 ml MeOH soxhle	7,469	22,6086	138,9084	n.a.	bMB	13115	02,12,21 15:02
247	LN 20 g Prop /2000 ml EtOH soxhlet	7,445	19,9491	74,2877	n.a.	BMB	6715	02,12,21 15:27

Figure S4. HPLC chromatogram of metanol extract of Çayeli propolis (Selected peak corresponds to caffeic acid).



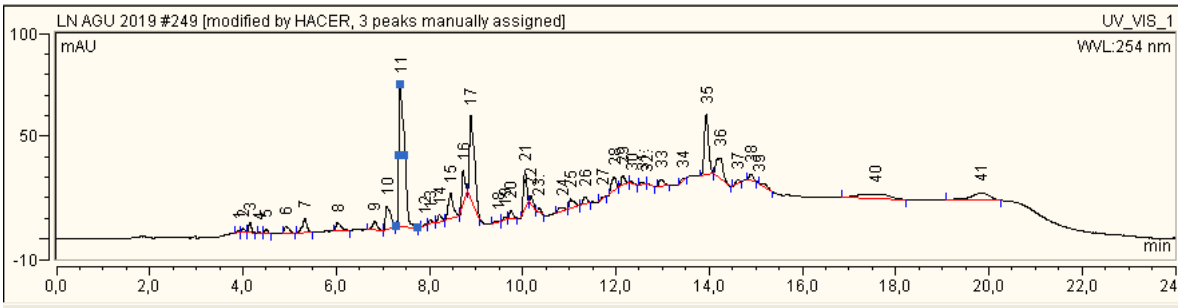
Sample No.	Sample Name	Ret.Time min [7.08 - 7.44] UV_VIS_1	Area mAU*min [7.08 - 7.44] UV_VIS_1	Height mAU [7.08 - 7.44] UV_VIS_1	Amount [7.08 - 7.44] UV_VIS_1	Type [7.08 - 7.44] UV_VIS_1	Plates (EP) [7.08 - 7.44] UV_VIS_1	Time
241	A.disli-propolis-Der Etoh2.	7,259	34,3825	114,2918	n.a.	BMB	4573	05,08,21 12:20

Figure S5. HPLC chromatogram of etanol extract of Çayeli propolis (Selected peak corresponds to caffeic acid).



Sample No.	Sample Name	Ret.Time min [16.89 - 17.76] UV_VIS_1	Area mAU*min [16.89 - 17.76] UV_VIS_1	Height mAU [16.89 - 17.76] UV_VIS_1	Amount [16.89 - 17.7 UV_VIS_1	Type [16.89 - 17.7 UV_VIS_1	Plates (EP) [16.89 - 17.76] UV_VIS_1	Time
237	A.disli-propolis-Sey Hegzan	17,323	5,0786	16,8513	n.a.	BMB	20491	04,08,21 15:47

Figure S6. HPLC chromatogram of hexane extract of Çayeli propolis (There is no caffeic acid peak in this chromatogram).



Sample No.	Sample Name	Ret.Time min [7.19 - 7.56] UV_VIS_1	Area mAU*min [7.19 - 7.56] UV_VIS_1	Height mAU [7.19 - 7.56] UV_VIS_1	Amount [7.19 - 7.56] UV_VIS_1	Type [7.19 - 7.56] UV_VIS_1	Plates (EP) [7.19 - 7.56] UV_VIS_1	Time
249	LN 20 g Prop /2250 mlSuda soxhlet ,	7,374	9,7013	69,3415	n.a.	BMB	14696	02,12,21 16:19

Figure S7. HPLC chromatogram of aquous extract of Çayeli propolis (Selected peak corresponds to caffeic acid).

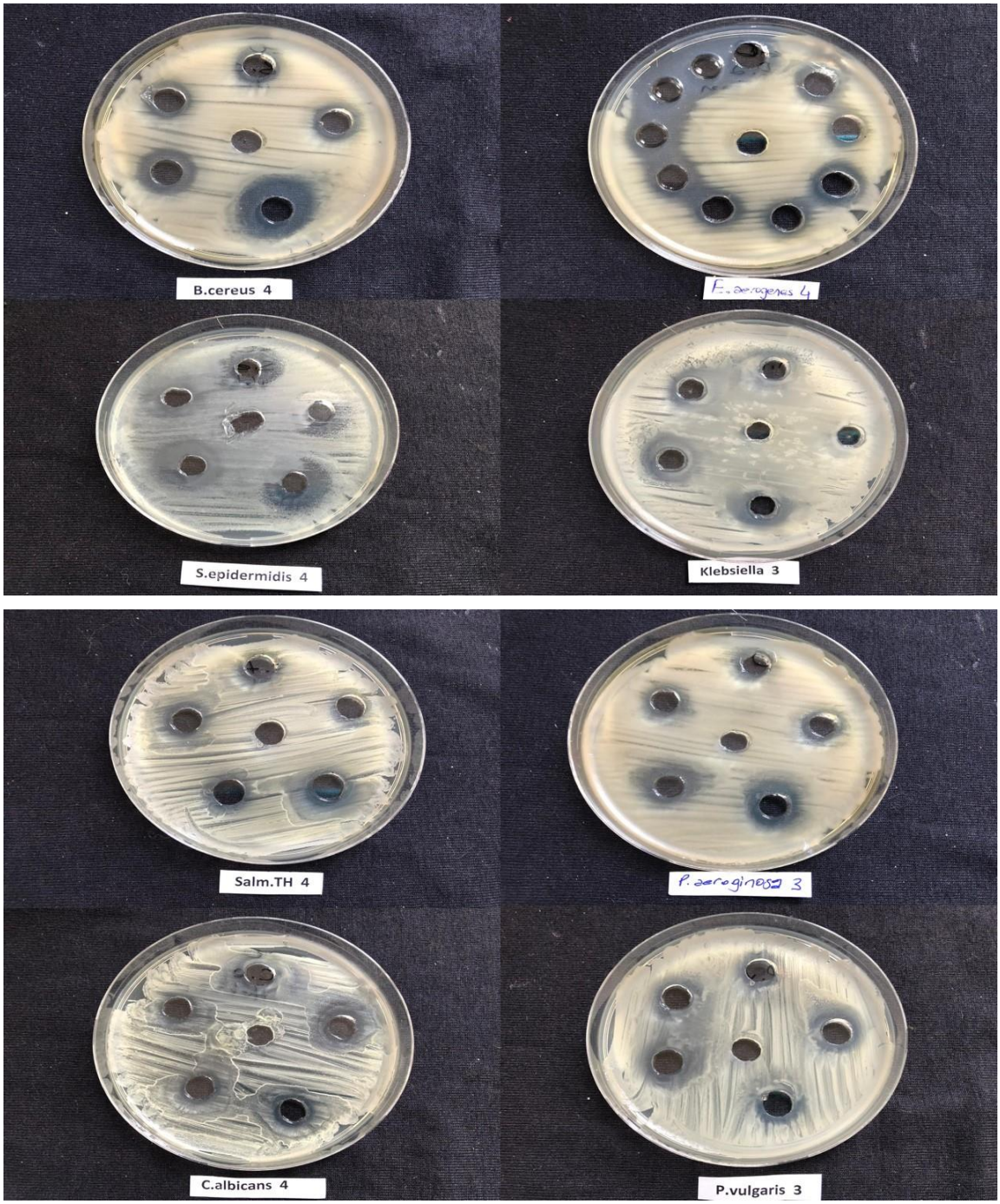


Figure S8. Selected photographs of the antimicrobial test plates

3. Tables

Table S1. Chemical constituents of ethanol extract analyzed by GC-MS

No	RT (min)	Name of the Compounds	Percentage of TIC
		Results of the ethanol extract without derivatization	
1	9.617	Benzoic acid	88.33
2	17.168	Dodecanoic acid	1.42
3	19.658	Hexadecanoic acid (palmitic acid)	2.03
		Results of the ethanol extract with derivatization	
1	11.538	Glycerol, 3TMS derivative	0.30
2	28.951	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	2.34
3	29.225	D-(-)-Fructose, pentakis(trimethylsilyl) ether, methyloxime (syn)	1.54
4	29.566	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	2.5
5	29.686	4-Coumaric acid, 2TMS derivative	0.14
6	29.967	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-	0.45
7	30.860	*muco*-Inositol, 6TMS derivative	0.26
8	32.143	Palmitic Acid, TMS derivative	0.19
9	32.291	Eicosane	1.02
10	32.993	Isoferulic acid, 2TMS derivative	0.39
11	33.067	2-Propenoic acid, 3-phenyl-, phenylmethyl ester, (E)-	0.54
12	34.451	Caffeic acid, 3TMS derivative	0.1
13	35.562	Dehydroabietic acid, TMS derivative	0.24
14	35.930	11-Octadecenoic acid, (E)-, TMS derivative	0.11
15	36.335	Tetracosane	2.44
16	36.508	Stearic acid, TMS derivative	0.19
17	37.598	Nonadecane	2.26
18	38.380	Isopimaric acid, TMS	0.26
19	39.485	Octadecane	4.53
20	43.702	Maltose, 8TMS derivative, isomer 1	2.97
21	44.349	Sucrose, 8TMS derivative	14.56
22	45.028	Lactulose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)	4.29
23	47.087	2-α-Mannobiose, octakis(trimethylsilyl) ether (isomer 1)	3.31

24	47.813	Tetracosanoic acid (lignoceric acid), TMS derivative	0.71
25	48.169	3-α-Mannobiose, octakis(trimethylsilyl) ether (isomer 2)	1.39

Table S2. Chemical constituents of methanol extract analyzed by GC-MS

No	**RT (min)**	**Name of the Compounds**	**Percentage of TIC**
		Results of the methanol extract without derivatization	
1	9.617	Benzoic acid	88.33
2	17.168	Dodecanoic acid	1.74
3	21.662	Octadecanoic acid (stearic acid)	1.61
		Results of the methanol extract with derivatization	
1	5.808	Cyclofenchene	0.058
2	8.675	Dodecane, 4-methyl-	1.412
3	8.764	Tridecane, 7-propyl-	0.312
4	10.014	(*5Z*)-10-methyl-6-(trifluoromethyl)-2-undeca-5,9-dienon	0.052
5	11.505	3-Hexanone, 4-ethyl-	0.041
6	12.158	Heptadecane, 2,6,10,15-tetramethyl-	0.123
7	12.395	(2S)-2-pentyl oxirane	0.042
8	14.549	Hexadecane, 1-iodo-	0.014
9	15.111	3-Hexanone, 4-ethyl-	0.011
10	17.238	Hexadecane, 1-iodo-	0.004
11	20.058	Hexadecane, 7,9-dimethyl-	0.002
12	24.900	Nonane, 3-methyl-5-propyl-	0.007
13	29.909	Tritetracontane	0.014

Table S3. Phenolic compounds of propolis in different solvents

Phenolic compounds	**Solvents**			
	Methanol (ppm±SD)	Ethanol (ppm±SD)	Hexane (ppm±SD)	Water (ppm±SD)
Gallic acid	n.d.	n.d.	n.d.	17.04±0.2
Caffeic acid	642.59±12.1	17.68±0.1	n.d.	275.00±10.6
Ferulic acid	359.66±8.4	95.30±2.3	20.89±0,4	29.15±1.1
Rutin	n.d.	n.d.	n.d.	1.18±0.01
Taxifolin	284.95±6.6	n.d.	52.69±1.1	22.42±0.5
Silycristin	207.86±4.5	39.68±0.1	85.68±1.6	19.44±0.1
Quercetin	168.85±18	100.00±1.7	n.d.	3.94±0.01
Silibinin	2749.20±12.1	27.10±0.2	42.72±0.8	56.31±1.7
Apigenin	n.d.	n.d.	10.69±0.1	3.97±0.02
Kaempferol	436.44±13.7	37.04±0.4	17.31±0.1	57.01±1.9

*n.d.: not detected.
**SD: Standard deviation

Table S4. Mineral contents of propolis extract in different solvents

Minerals	**Solvents**				
	Methanol (µg/g±SD)	Ethanol (µg/g±SD)	Hexane (µg/g±SD)	Water (µg/g±SD)	Raw propolis (µg/g±SD)
Zn	15.4 ±0.4	19.6±0.5	8.8±0.3	23.0±0.8	26.8±0.9
Na	615 ± 12	613±14	860±25	216±9	299±10
Mg	1100 ± 30	580 ± 20	480 ± 17	2.6 ± 0.3	14 ± 1
Ca	567 ± 14	232 ± 10	145 ± 13	298 ± 13	384 ± 13
K	628 ± 22	255 ± 16	<LOD	<LOD	1321.63

*LOD: limits of detection
**SD: Standard deviation

Table S5. Antimicrobial activity of propolis extracts against Gram-positive and Gram-negative bacteria and a yeast strain

		Inhibition zone diameter (mm)							
Extracts	Concentrations (ppm)	*S. epidermidis*	*B. cereus*	*C. albicans*	*P. aeruginosa*	*K. pneumonia*	*E. aerogenes*	*S. typhi*	*P. vulgaris*
AEP	0.1	-	16	12	17	15	20	16	-
	0.5	15	16	12	16	17	20	16	18
	1.0	20	17	15	20	18	20	17	20
	1.5	24	17	16	20	17	22	17	21
	2.0	26	17	15	20	17	22	17	21
EEP	0.1	-	15	16	14	13	20	13	16
	0.5	-	15	18	16	13	19	15	18
	1.0	-	15	18	16	17	17	13	19
	1.5	-	15	18	18	14	17	13	17
	2.0	-	14	18	17	21	18	13	16
MEP	0.1	15	16	15	20	18	18	15	16
	0.5	16	16	25	20	11	20	15	21
	1.0	16	30	25	21	21	18	16	20
	1.5	17	18	20	22	21	20	17	22
	2.0	17	17	25	20	22	19	17	23
HEP	0.1	23	20	26	12	15	20	15	-
	0.5	21	16	28	18	17	20	16	18
	1.0	25	20	22	20	13	17	20	22
	1.5	24	20	22	19	13	20	17	20
	2.0	25	20	22	14	12	20	20	18
Commercial agents	Ampicillin	26	23	-	8	21	21	11	17
	Sulfamethoxazole	25	25	-	18	20	19	17	19
	Amoxicillin	27	20	-	15	21	20	19	20
	Kanamycin	25	28	-	14	23	24	20	21
	Nystatin	-	-	20	-	-	-	-	-

Control groups	Water	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-
	Hexane	-	-	-	-	-	-	-	-

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