Journal of Apicultural Research

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/tjar20

Chemical composition and biological activities of Cypriot propolis

Ayşe Nalbantsoy, Nazli Boke Sarıkahya, Cenk Serhan Özverel, Ayşe Berçin Barlas, Damla Kırcı, İsmail Hakkı Akgün, Tansel Yalçın, Gamze Düven, Duygu Kışla, Betül Demirci, Gulacti Topcu & Ahmet C. Goren

To cite this article: Ayşe Nalbantsoy, Nazli Boke Sarıkahya, Cenk Serhan Özverel, Ayşe Berçin Barlas, Damla Kırcı, İsmail Hakkı Akgün, Tansel Yalçın, Gamze Düven, Duygu Kışla, Betül Demirci, Gulacti Topcu & Ahmet C. Goren (2022) Chemical composition and biological activities of Cypriot propolis, Journal of Apicultural Research, 61:2, 233-245, DOI: 10.1080/00218839.2021.1977028

To link to this article: https://doi.org/10.1080/00218839.2021.1977028

JOURNAL OF APICULTURAL RESEARCH
2022, VOL. 61, NO. 2, 233–245
https://doi.org/10.1080/00218839.2021.1977028

ORIGINAL RESEARCH ARTICLE

Chemical composition and biological activities of Cypriot propolis

Ayşe Nalbantsoy[a]*, Nazli Boke Sarıkahya[b], Cenk Serhan Özverel[c†], Ayşe Berçin Barlas[a], Damla Kırcı[d], İsmail Hakkı Akgün[a], Tansel Yalçın[c], Gamze Düven[e], Duygu Kışla[f], Betül Demirci[d], Gulacti Topcu[g] and Ahmet C. Goren[h,i]*

[a]Department of Bioengineering, Faculty of Engineering, Ege University, Izmir, Turkey; [b]Department of Chemistry, Faculty of Science, Ege University, Izmir, Turkey; [c]Department of Biology, Faculty of Science, Ege University, Izmir, Turkey; [d]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey; [e]Department of Food Engineering, Faculty of Engineering, Izmir Institute of Technology, Izmir, Turkey; [f]Department of Food Engineering, Faculty of Engineering, Ege University, Izmir, Turkey; [g]Department of Pharmacognosy, Faculty of Pharmacy, Bezmialem Vakif University, Istanbul, Turkey; [h]Department of Analytical Chemistry, Faculty of Pharmacy, Bezmialem Vakif University, Istanbul, Turkey; [i]Drug Application and Research Center (İLMER), Bezmialem Vakif University, Istanbul, Turkey

ABSTRACT

Propolis compositions are highly variable, depending on the geographic region and the season of collection. In this study, propolis samples from seven different regions of Cyprus were studied for the first time by means of chemical content and biological activities. Secondary metabolite composition was determined by LC-HRMS. While the major flavonoids found were isosakuranetin, naringenin, rhamnocitrin, diosmetin, chrysin and acacetin, interestingly verbascoside, a phenylethanoid glycoside, and chlorogenic acid were identified as the major compounds in the ethanol-water extracts. α-Pinene was detected as the major compound of propolis extracts according to the volatile compositions via GC-MS. Karaoglanoglu and Tirmen extracts, presenting different chemical profiles, exerted enormous cytotoxic activity by MTT assay (IC_{50}: 2.36–11.56 μg/mL; 1.44–9.33 μg/mL, respectively). The highest iNOS inhibition potential was detected in the Karpaz extract (IC_{50}:2.6 μg/mL) in LPS induced RAW 264.7 cells whereas the Guzelyurt sample demonstrated remarkable antioxidant (88.82 ± 0.10%) and antimicrobial activities (with a MIC value of 31.2 μg/mL against *S. aureus, S. epidermidis, E. faecium*, and *E. faecalis*).

ARTICLE HISTORY
Received 29 December 2020
Accepted 3 August 2021

KEYWORDS
Propolis; Cyprus; LC-HRMS; GC-MS; biological activities

Introduction

Propolis is a resinous material collected by bees from different parts of plants including buds, exudates, branches, and barks. Bees collect these resinous materials, add their salivary secretions and enzymes to form a rigid material for protection against microorganisms and insects (Castaldo & Capasso, 2002; Tiveron et al., 2016). Its chemical nature is formed from resins (50%), waxes (30%), essential oils (10%), pollens (5%) and organic compounds (5%) (Burdock, 1998; Castaldo & Capasso, 2002; Park et al., 2002; Wagh, 2013). Propolis is known for its highly variable composition, depending on the collection area and the season of collection (De Vecchi & Drago, 2007). These all make propolis collected from different locations unique in composition and biological activities (Kujumgiev et al., 1999). Propolis is considered a potential candidate for drug and natural food supplement due to its antimicrobial, anticancer, antiinflammatory, antioxidant, immunostimulatory, anesthetic and cytotoxic effects (Bankova et al., 2000; Ishida et al., 2018).

The biological activities of propolis come from its complex secondary metabolite ingredients including terpenes, pterocarpans, prenylated benzophenones, and especially phenolic compounds (Rufatto et al., 2017). Flavonoids, one of the secondary metabolites of propolis, are known for their activities in nitric oxide inhibition via acting as free radicals scavengers that are generated by macrophages and neutrophils (Blonska et al., 2004). Free radical scavengers and antioxidants are important for general health as excessive free radical production and lipid peroxidation and are strong inducers of cancer, atherosclerosis, and chronic inflammatory diseases (Chu et al., 2000). The phenolic contents of propolis exert various mechanisms of action such as apoptosis induction, mitochondrial stress induction, cancer cell proliferation inhibition, and cell cycle arrest (Benguedouar et al., 2008). Propolis is also known for its immunomodulatory and antiinflammatory

CONTACT Ayşe Nalbantsoy analbantsoy@gmail.com; Ahmet C. Goren ahmet.goren@acgpubs.org
[†]Current address: Near East University, Research Center of Experimental Health Sciences, Near East Boulevard, 99138 Nicosia, Cyprus.
Supplemental data for this article is available online at https://doi.org/10.1080/00218839.2021.1977028.

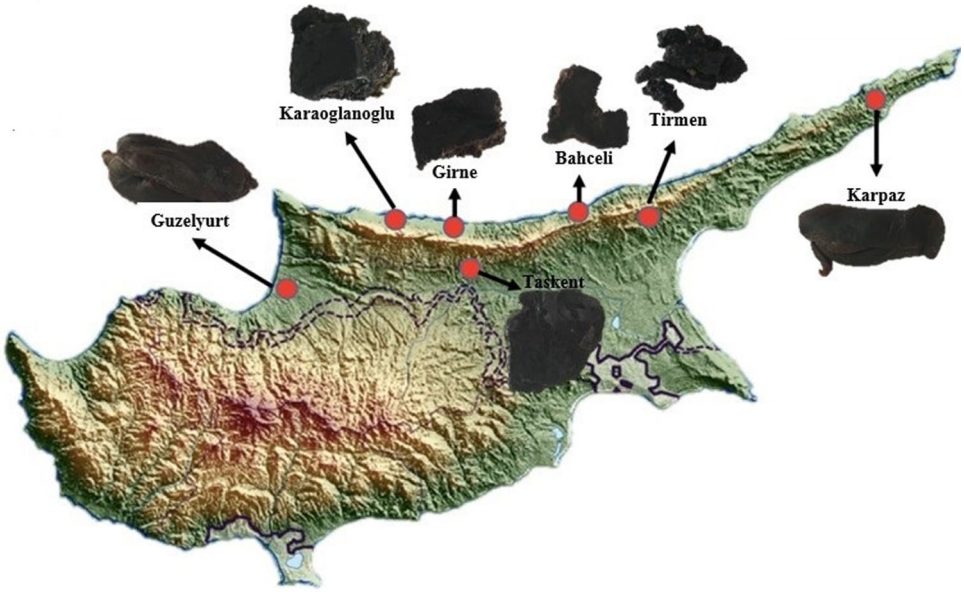

Figure 1. Collected propolis samples region from various geographical regions of Cyprus.

potentials. Inflammation is a host innate process that recruits effector cells and mediators to the site of infection (Bueno-Silva et al., 2017).

In this study, a comparative study was performed on propolis samples collected from seven different geographical regions of Cyprus for cytotoxic, iNOS inhibition, antioxidant and antimicrobial activities. Besides that, the secondary metabolites and volatile composition of samples were determined by LC-HRMS and GC-MS, respectively. HPLC fingerprint was also analyzed. To the best of our knowledge, this is the first study in the literature performed on Cypriot propolis samples.

Materials and methods

Materials

In this study, propolis samples from seven different geographical were collected (Figure 1). The collected samples were kept in a dark place at +4 °C until being used. Six propolis samples Bahceli (**1**), Karaoglanoglu (**2**), Girne (**3**), Tirmen (**4**), Guzelyurt (**5**), and Karpaz (**6**) were collected from the Northern Cyprus Beekeepers Foundation upon our request and propolis sample from Taskent (**7**) was generously donated by local beekeepers living in Taskent Village.

To give brief information about the botanical origins of the samples; mainly forest, maquis trees, and seasonal plants affect the content of the propolis depending on the region where they were collected. The propolis samples belonging to Girne, Tirmen, and Bahceli were collected from the beehives in the forest area, samples from Taskent, Guzelyurt and Karpaz were collected from beehives in the maquis area. In the island where the Mediterranean climate prevails, the most common tree species is red pine (*Pinus brutia*). Cypress species (*Cupressus sempervirans*) is the second most common tree species. Peanut pine (*Pinus pinea*), almond (*Prunus dulcis*), and Aleppo pine (*Pinus halepensis*) are also species seen in the forest formation. Apart from these, juniper (*Juniperus squamata*) is also common and citrus trees are also significantly present in some areas such as Guzelyurt (Chapman, 1980; Ilseven, 2017). In the maquis area, the main scrub elements are olives (*Olea europaea*), mastic (*Pistacia lentiscus, Pistacia terebinthus*), daphne (*Laurus nobilis*), myrtle (*Myrtus communis*), sandal wood (*Arbutus andrachne*), carob (*Ceratonia siliqua*) and the kermes oak (*Quercus coccifera, Quercus infectoria*) (Ilseven, 2017). In addition, hundreds of endemic and non-endemic seasonal plants such as anemones, rock roses, orchids, tulips, chamomile, thyme, jasmine, iris, narcissi, etc. are common seasonal plants of Northern Cyprus (Meikle, 1985).

Chemicals

One hundred milligrams/liter dihydrocapsaicin (97%, Sigma-Aldrich) solution was freshly prepared as stock solution was used as an internal standard (IS). Following compounds were used as standards for method validation in LC-HRMS analysis: (+)-*trans* taxifolin (>97%, TRC Canada), 3-*O*-methylquercetin (>97%, TRC Canada), acacetin (>97%, TRC Canada), apigenin-7-glucoside (>97%, EDQM CS), apigenin (>97%, TRC Canada), caffeic acid (98%, Sigma-Aldrich), chrysin (≥96%, Sigma-Aldrich), dihydrokaempferol (>97%, Phytolab), ellagic acid (>97%, TRC Canada), eupatilin (98%, Sigma-Aldrich), fumaric acid (≥99%, Sigma-Aldrich), hederagenin (>97%, TRC

Canada), hesperidin (>98%, J&K scientific ltd GmbH), hispidulin (>97%, TRC Canada), hyperoside (>97%, TRC Canada), isosakuranetin (>97%, Phytolab), luteolin-7-rutinoside (>97%, Carbosynth limited), myricetin (>95%, Carl Roth GmbH + Co), naringenin (≥95%, Sigma-Aldrich), naringin (≥90%, Sigma-Aldrich), nepetin (98%, Supelco), nepetin-7-glucoside (>97%, Phytolab), quercetin (≥95%, Sigma-Aldrich), rhamnocitrin (>97%, Phytolab), rutin (≥99%, Sigma-Aldrich), verbascoside (86.31%, Hwi Analytik Gmbh), diosmetin (95%, Sigma-Aldrich), chlorogenic acid (95%, Sigma-Aldrich), quercetin-3-*O*-rutinoside (≥95.0% Sigma-Aldrich), *p*-coumaric acid (≥99.0% Sigma-Aldrich), *trans*-ferulic acid (≥99.0% Sigma-Aldrich), oleanoic acid (95% isolated from our previous work) (Sarikahya et al., 2019), tormentic acid (>97%, isolated from our previous work) (Dagli et al., 2019).

Preparation of water-ethanol extracts of propolis

Propolis extracts were prepared according to Trusheva et al. (2007) with minor modifications. Propolis samples weighted as 1 g and 70% aqueous ethanol (Alkomed, Turkey) solution was added on the samples with a total of 10 mL volume. All the samples were sonicated four times in ultrasonic water bath (Ultrasonic LC30, Elma, Germany) at room temperature for 1 hour. After extraction, solid propolis and liquid extracts were separated by centrifuging at 4100 rpm for 5 minutes. The solution was filtered through a 0.45 μm Chrom Fil PTFE-L filter and each sample was combined and ethanol was evaporated at 40 °C under vacuum. Dried extracts were then lyophilized for 24 h. Samples were kept in dark at +4 °C until the analysis of cytotoxicity screening, iNOS inhibition, antioxidant, antimicrobial activities, LC-HRMS, HPLC-DAD profiling, and HCA analysis. For all biological tests, propolis samples were dissolved with cell culture-specific DMSO (Sigma-Aldrich) and DMSO was used at nontoxic concentration (0.5 to 0.005%) as a negative control in the tests (Nguyen et al., 2020).

Preparation of samples for LC-HRMS analysis

Fifty to one hundred milligrams of the ethanol-water extracts of propolis were added to 5 mL volumetric flasks and the extracts were dissolved in distilled water. The flask was exposed to ultrasonication for 20 min in an ultrasonic bath. Then, 100 μL of freshly prepared internal standard solution (dihydrocapsaicin) from 100 ppm stock solution was added, diluted to the volume with mobile phase A and B mixture (1:1) and vortexed for 20 seconds. The mixture was heated warmly to get a clear solution, if necessary. Then the final solution was filtered by using a 0.45 μm Millipore Millex-HV filter. From the final solution, 1 mL was transferred to an autosampler vial and 2 μL of sample was injected to LC-MS for each measurement. The temperature of the autosampler vials was set to at 15°C during the experiment (Hamad et al., 2017; Sarikahya et al., 2019).

Instruments and chromatographic conditions of LC-HRMS

LC-HRMS experiments were performed on a Thermo ORBITRAP Q-EXACTIVE mass spectrometry equipped with a Fortis C18 column (150 × 3 mm i.d., 3 μm particle size). The mobile phase was composed of 1% formic acid-water for A and 1% formic acid-methanol for B. The gradient programme of which was 0–1.00 min 50% A and 50% B, 1.01–6.00 min 100% B and finally 6.01–10 min 50% A and 50% B. The flow rate of the mobile phase was 0.35 mL/min, and the column temperature was set to 22 °C. Environmental conditions were set as temperature 22.0 ± 5.0 °C and relative humidity (50 ± 15) % rh (Hamad et al., 2017).

Optimization of HPLC methods and LC-HRMS procedure

According to our former experiences on the measurement of plant derived extracts (Hamad et al., 2017, Sarikahya et al., 2019), we decided to use a gradient of acidified methanol and water mobile phase system chromatographic separation of the secondary metabolites. ESI (electrospray ionization) source was selected for ionization of the metabolites due to good ionization patterns of it for polar secondary metabolites such as simple phenolics, flavonoids, triterpenoids and saponins. Regarding expected chemical composition of secondary metabolites of the propolis extracts, the scan range of the ions is chosen as m/z 85–1500. Retention time of standard compounds (in the range of purity 95%–99% see section chemicals) and HRMS data of Bezmialem Vakif University, Drug Application and Research Center Library (ILMER) were used for identification of secondary metabolites of the extracts. Dihydrocapsaicin (purity 97%) was used as an internal standard in the validated method. The detailed mass parameter of each target compound was given in Table S1 (Supplementary material).

HPLC-DAD profiling of propolis samples

Dissolved sample extracts in methanol (10 mg/mL) were analyzed using HPLC-DAD machine equipped with Thermo Accela PDA detector, autosampler (Thermo Fischer Scientific, Germany) and Shimadzu

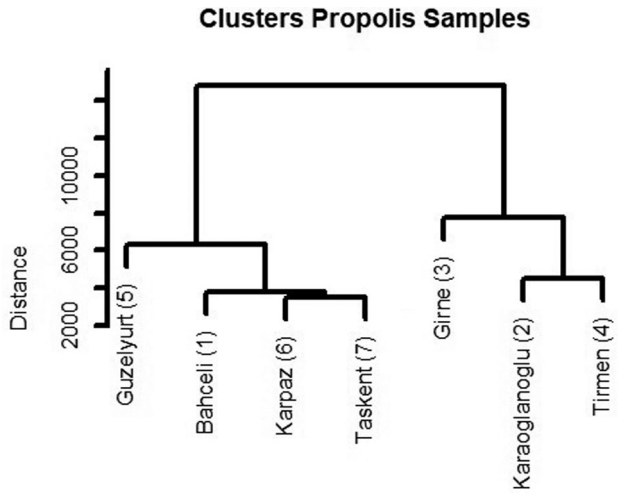

Figure 2. Hierarchical cluster analysis of Cypriot propolis samples.

LC-10AT pump (Shimadzu, Kyoto, Japan). Analysis of samples was carried out using water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as eluents on a Thermo Hypersil Gold C18 column with 100 mm × 4.6 mm i.d. × 5 μm particle size. Separation of the molecules in the samples was carried out following analysis program: 0–4 min, 25 ∼ 25% B; 5–45 min 25 ∼ 95% B, 45–50 min 95 ∼ 95% B, 50–60 min 25 ∼ 25% B. The detection wavelength was set 254 nm and UV – VIS spectrum of the samples was monitored between 200 and 600 nm with 1 Hz sampling rate. The injection volume of the samples was 10 μL, and the column temperature was set to 30 °C. Hierarchical Cluster Analysis (HCA) of the samples was carried out using HPLC chromatogram of the samples between 0 and 50 min at 254 nm with R software (Figure 2).

Headspace-SPME

The manual SPME device (Supelco, Bellafonte, PA, USA) with a fiber-precoated 65 μm (PDMS/DVB-blue) was used for extraction of sample volatiles according to Tasdemir et al. (2003).

Analysis of volatile compounds

The volatiles were analyzed by GC-MS using an Agilent 5975 GC-MSD system. The column, carrier gas, GC programme, injector temperature and Mass analysis conditions were set according to Tasdemir et al. (2003). Identification of the volatile components was carried out by commercial libraries Wiley and Mass Finder Software 4.0 (Hochmuth, 2008; McLafferty & Stauffer, 1989) and in-house "Baser Library of Essential Oil Constituents" built up by uniquie components of identified essential oils. Relative percentage amounts of the separated compounds were calculated from Total Ion Chromatograms (TIC).

Cell lines and maintenance

Cancerous PANC-I (pancreas); MDA-MB-231 (breast); MCF-7 (breast); Hela (cervix); CaCo-2 (colon); PC-3 (prostate); A549 (lung); SHSY5Y (neuroblastoma) and non-cancerous HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium F12 (Serox, Mannheim, Germany), supplemented with 10% fetal bovine serum (Serox, Mannheim, Germany), 2 mM glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Cytotoxicity assay

Cytotoxicity study was performed by using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide)] assay (Mossman, 1983; Onbas et al., 2016). Doxorubicin was used as a positive control. All data were studied as triplicates and data are presented as mean ± standard error of mean (SEM) of samples. The viability (%) was estimated using the following equation:

%Viable cells = [(absorbance of treated cells) – (absorbance of blank)]/[(absorbance of negative control) – (absorbance of blank)] × 100.

Determination of half maximal inhibitory concentration (IC_{50})

In cell culture studies for DMSO treated cells (negative controls) cytotoxicity was set to 0%. The IC_{50} values were calculated by fitting the data to a sigmoidal curve and using a four parameter logistic model and presented as an average of three independent measurements. The IC_{50} values were reported at 95% confidence interval and calculations

were performed using Prism 5 software (GraphPad5, San Diego, CA, USA).

Nitric oxide analysis

RAW 264.7 cells were cultured in RPMI 1640 medium were seeded in 96-well plates (1×10^5 cells/mL) and incubated for 24 h. RAW 264.7 cells were induced with lipopolysaccharide (1 μg/mL) and different concentrations of samples were added at the same time and incubated for another 24 h. All the samples were tested in triplicate. The level of nitrite in the medium was measured by using Griess reagent in supernatants at 540 nm (Onbas et al., 2016; Quang et al., 2006). Percent inhibition of nitrite production by the sample was calculated in comparison to vehicle control and IC_{50} values were obtained from dose curves using Graph Pad Prism 5.0 software (San Diago, USA).

Determination of scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The measurement of scavenging activity of propolis extracts on DPPH radicals (Sigma Chemical Co., St Lois, MO, USA) was performed according to Yan-Hwa et al. (2000) with some modifications. An aliquot of 0.5 mL propolis extracts in DMSO (1 mg/mL) and 1 mL of 0.1 mM DPPH in methanol (Merck, Germany) were mixed thoroughly (vortex for 1 min). The mixture was incubated for 20 min at room temperature and the absorbance at 520 nm was measured (Thermo Fisher Scientific Genesys, MA, USA). Samples were tested in triplicate and methanol was used as a blank solution. The scavenging activity on DPPH radicals was calculated with the equation below.

$$\text{DPPH scavenging activity } (\%) = 1 - \frac{\text{Absorbance at 520 nm in presence of sample}}{\text{Absorbance at 520 nm in absence of sample}}$$

Microorganisms and antimicrobial assay

The antimicrobial activity of different propolis samples was evaluated against Gram-negative enteropathogenic *Escherichia coli* O157:H7 (RSKK 234), *Salmonella* typhimurium CCM 5445 and *Pseudomonas aeroginosa* ATCC 27853 and Gram-positive bacterial strains *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecium* DSM 13590, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 12228 and *Listeria monocytogenes* ATCC 19111. Antifungal activity of the propolis samples was also tested on *Candida albicans* ATCC 10239 and *C. tropicalis* RSKK. The lyophilized pure bacterial and yeast strains were provided by Ege University, Faculty of Science, Department of Basic and Industrial Microbiology (Izmir, Turkey). Minimum Inhibitory Concentration (MIC) values of different propolis samples were determined by broth micro-dilution technique (microdilution technique) according to the CLSI (CLSI, 2009). Gentamicin and flucytosine were, respectively used as standard antibacterial and antifungal agents. Microbial strains were grown to exponential phase in Mueller-Hinton (MH) broth (Oxoid, Hampshire, England). Then, the cell density of each reference strain suspension was adjusted to 0.5 McFarland standard (1.5×108 CFU/mL). Serial dilutions of the propolis samples were prepared. Eighty microliters of sample from each dilution was transferred into 96 well sterile microtitre plates and 20 μL of the microbial inocula were then added to obtain a final volume of 100 μL in each well. Microbial growth was assessed by eye inspection. MH broth and MH broth inoculated with test microorganisms were employed as negative and positive control, respectively. MIC was defined as the lowest concentration of the propolis samples required to inhibit microbial growth after 24 h. Minimum Bactericidal Concentrations (MBC) and Minimum Fungicidal Concentrations (MFC) values of the tested propolis samples were evaluated by sub-culturing about 5–10 μl of the samples in wells with a concentration equal or higher than MIC on MH agar plate for microorganisms. The lowest concentration that did not show bacterial growth was defined as the MBC value. The lowest concentration that did not show fungal growth was defined as the MFC value. Samples were tested triplicate and the results were expressed in μg/mL.

Results

In this study, seven different propolis samples collected from different geographic regions of Cyprus were performed for cytotoxicity, iNOS inhibition, antioxidant, antimicrobial activities. Moreover, secondary metabolites and volatile compounds composition were analyzed by LC-HRMS and GC-MS, respectively. HPLC fingerprint analyses were also studied.

LC-HRMS analysis

The liquid chromatography and high-resolution mass spectrometry method were adopted and validated to analyze the chemical constituents of propolis samples from Cyprus. The identification of the analytes by means of high-resolution mass spectrum (HRMS) of each compound was determined together with retention times of them in Fortis C18 column using mobile phase A and mobile phase B (Table 1 and Supplementary material, Figure S1).

Table 1. The volatile components of Cypriot propolis samples were characterized by HS-SPME coupled with GC-MS system.

RRI	Compound	1	2	3	4	5	6	7	IM
1032	α-Pinene	55.6	66.8	34.2	51.3	63.3	57.6	45.1	RRI, MS
1093	Hexanal	–	–	6.6	–	–	–	–	RRI, MS
1118	β-Pinene	–	1.5	–	5.1	2.7	2.4	0.5	RRI, MS
1132	Sabinene	–	0.2	–	1.4	3.7	3.0	22.8	RRI, MS
1151	δ-3-Carene	2.1	2.4	–	–	–	–	–	MS
1176	α-Phellandrene	–	–	–	–	0.1	–	–	RRI, MS
1188	α-Terpinene	–	1.0	–	–	2.2	1.8	–	RRI, MS
1203	Limonene	–	6.5	2.9	17.6	2.4	2.4	2.6	RRI, MS
1218	β-Phellandrene	–	0.6	–	0.8	–	0.3	–	RRI, MS
1255	γ-Terpinene	1.0	–	–	–	1.6	1.3	0.9	RRI, MS
1280	*p*-Cymene	2.7	5.3	4.8	3.0	5.1	4.8	3.6	RRI, MS
1290	Terpinolene	–	–	–	–	0.4	0.2	–	RRI, MS
1296	Octanal	4.5	–	3.6	–	–	–	–	MS
1348	6-Methyl-5-hepten-2-one	–	0.6	2.4	1.0	0.1	0.2	–	MS
1360	1-Hexanol	–	–	0.6	–	–	–	–	MS
1400	Nonanal	7.3	–	3.2	0.8	0.2	0.3	0.4	MS
1441	*(E)*-2-Octanal	–	–	0.8	0.3	–	–	–	MS
1443	*2,5*-Dimethyl styrene	–	–	0.4	–	0.1	0.1	0.3	MS
1452	*α,p*-Dimethyl styrene	0.5	0.5	1.0	0.4	0.9	0.8	0.8	MS
1452	1-Octen-3-ol	–	0.5	–	0.8	–	tr	0.2	MS
1463	1-Heptanol	–	–	0.2	–	–	–	–	MS
1466	α-Cubebene	0.6	0.6	–	–	0.6	0.7	0.2	MS
1467	6-Methyl-5-hepten-2-ol	–	0.1	1.1	0.6	–	tr	0.1	MS
1474	*trans*-Sabinene hydrate	0.4	0.3	tr	–	0.2	0.4	0.2	MS
1479	Furfural	0.4	–	0.8	–	0.3	0.3	0.3	RRI, MS
1493	α-Ylangene	–	0.1	–	–	–	–	–	MS
1496	2-Ethyl hexanol	–	–	0.4	0.8	–	–	–	MS
1497	α-Copaene	–	1.2	–	0.2	0.2	0.2	–	MS
1498	2-Propenoic acid, 6-methylhepthyl ester (=*2-Ethylhexyl acrylate*)*	–	–	3.5	–	–	–	–	MS
1499	α-Campholene aldehyde	–	0.2	–	–	0.1	0.2	–	MS
1506	Decanal	3.8	–	1.3	–	–	–	–	MS
1532	Camphor	0.3	0.1	0.7	0.4	0.3	0.5	0.7	RRI, MS
1535	β-Bourbonone	–	0.3	–	0.1	tr	0.1	–	MS
1536	Pinocamphone	–	0.1	–	–	–	–	–	RRI, MS
1541	Benzaldehyde	–	–	1.1	0.2	–	0.1	0.3	RRI, MS
1553	Linalool	–	0.4	–	0.4	0.1	0.1	0.1	RRI, MS
1556	*cis*-Sabinene hydrate	0.2	0.1	–	0.1	0.1	0.2	–	MS
1562	Isopinocamphone	–	–	–	–	–	tr	–	RRI, MS
1562	Octanol	0.1	Tr	0.7	0.2	–	–	0.1	RRI, MS
1583	Longifolene (=*Junipene*)	1.0	0.6	–	0.6	1.6	1.9	–	MS
1586	Pinocarvone	0.5	0.3	0.4	0.2	–	–	0.5	RRI, MS
1590	Bornyl acetate	1.2	0.4	1.6	0.8	1.1	1.5	1.3	RRI, MS
1591	2-Methyl propanoic acid	–	–	0.5	–	–	–	0.1	MS
1594	1,7-*diepi*-β-Cedrene	–	–	–	–	0.5	0.9	–	MS
1597	β-Copaene	–	0.2	–	–	–	–	–	MS
1601	Nopinone	0.1	–	0.5	–	0.2	0.3	0.3	MS
1611	Terpinen-4-ol	1.8	0.5	0.3	0.7	–	–	–	RRI, MS
1612	β-Caryophyllene	–	1.8	0.5	0.5	–	–	–	RRI, MS
1613	β-Cedrene	0.2	–	–	–	0.3	0.3	0.3	MS
1614	Carvacrol methyl ether	1.5	0.3	1.5	–	4.2	5.3	7.3	RRI, MS
1625	4,4-Dimethyl but-2-enolide	–	0.1	0.8	0.3	–	0.1	0.1	MS
1630	4-Terpinenyl acetate	–	–	–	–	0.1	0.2	0.2	MS
1648	Myrtenal	0.1	0.1	0.3	0.1	0.2	0.3	0.3	MS
1651	γ-Butyrolactone	0.1	–	0.3	–	–	–	–	MS
1651	Sabinaketone	–	–	–	–	0.1	0.1	–	MS
1670	*trans*-Pinocarveol	1.6	0.6	3.2	1.1	1.1	1.7	1.6	RRI, MS
1683	*trans*-Verbenol	0.7	0.4	1.4	0.6	0.4	0.7	–	RRI, MS
1687	α-Humulene	–	0.1	–	–	–	–	–	RRI, MS
1700	*p*-Mentha-1,8-dien-4-ol (=*Limonen-4-ol*)	0.3	–	0.2	–	0.3	0.5	0.2	RRI, MS
1704	γ-Muurolene	–	0.3	–	0.2	–	–	–	MS
1706	α-Terpineol	0.4	0.3	0.8	0.2	0.3	0.5	0.3	RRI, MS
1709	α-Terpinyl acetate	0.5	0.1	0.1	0.3	0.4	0.7	0.6	RRI, MS
1719	Borneol	0.7	0.1	1.6	0.2	0.4	0.9	0.8	RRI, MS
1725	Verbenone	0.2	0.1	0.3	0.1	0.2	0.4	0.3	RRI, MS
1740	α-Muurolene	–	0.1	–	–	tr	0.1	0.1	MS
1773	δ-Cadinene	–	0.1	–	0.1	0.1	0.1	0.1	MS
1776	γ-Cadinene	–	0.1	–	–	–	–	–	MS
1804	Myrtenol	0.2	0.1	0.6	0.2	0.2	0.3	0.4	MS
1845	*trans*-Carveol	0.1	–	–	–	–	0.2	0.1	RRI, MS
1849	Calamenene	–	0.2	–	–	0.1	0.1	0.1	MS
1856	*m*-Cymen-8-ol	–	–	–	–	0.1	0.1	0.3	MS
1864	*p*-Cymen-8-ol	0.2	0.1	0.3	0.2	0.2	0.4	0.4	MS
1896	BPharmaenzyl alcohol	–	–	–	0.1	–	–	tr	RRI, MS
1925	Ionol	0.6	0.5	0.7	1.2	0.1	0.2	0.2	RRI, MS
1935	Phenyl ethyl alcohol	0.1	–	–	–	tr	tr	0.1	RRI, MS
2143	Cedrol	1.4	0.1	4.1	0.1	0.6	1.2	0.7	RRI, MS

(*continued*)

Table 1. Continued.

RRI	Compound	1	2	3	4	5	6	7	IM
2239	Carvacrol	–	–	–	0.3	–	0.1	0.1	RRI, MS
2400	Tetracosane	0.1	–	–	–	0.1	–	–	RRI, MS
2500	Pentacosane	0.3	–	–	–	0.2	–	–	RRI, MS
2600	Hexacosane	0.5	–	–	–	tr	–	–	RRI, MS
2700	Heptacosane	0.5	–	–	–	–	–	–	RRI, MS
2800	Octacosane	0.6	–	–	–	–	–	–	RRI, MS
2900	Nonacosane	0.5	–	–	–	–	–	–	RRI, MS
	Total:	96.5	96.0	90.3	93.6	97.8	97.1	96.0	

Volatile components were identified by making comparisons to their retention times to series of *n*-alkanes or authentic samples. Percentages of the separated compounds were calculated by using TIC. The origins of propolis extracts are abbreviated with numbers as follows **1**: Bahceli; **2**: Karaoglanoglu**; 3**: Girne; **4**: Tirmen; **5**: Güzelyurt; **6**: Karpaz; **7**: Taskent.
*tentative identification from Wiley.

Table 2. Compounds and their amounts (mg/g extract) in ethanol-water extracts of propolis from Cyprus.

Compounds	1	2	3	4	5	6	7
Trans-taxifolin	0.01	<LOD	<LOD	<LOD	<LOD	<LOD	0.02
3-*O*-Methylquercetin	0.17	0.25	0.54	0.50	0.30	0.40	1.33
Acacetin	0.35	0.62	3.10	1.39	8.48	9.36	2.63
Apigenin 7-glucoside	<LOD	0.09	<LOD	<LOD	<LOD	<LOD	<LOD
Apigenin	0.01	0.22	1.68	0.61	2.05	0.16	0.03
Caffeic acid	0.10	<LOD	0.28	0.73	<LOD	<LOD	0.13
Chrysin	0.32	0.74	3.66	1.63	13.79	8.74	1.00
Dihydrokaempferol	0.08	<LOD	0.03	0.20	<LOD	<LOD	0.18
Ellagic acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.01
Eupatilin	0.76	3.39	3.43	2.18	1.87	1.31	2.97
Fumaric acid	0.96	<LOD	<LOD	<LOD	<LOD	<LOD	0.34
Hederagenin	5.25	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Hesperidin	0.20	<LOD	<LOD	<LOD	<LOD	5.52	<LOD
Hispidulin	0.36	1.11	1.64	1.77	3.25	4.09	3.89
Hyperoside	1.91	0.26	3.41	5.14	<LOD	<LOD	3.07
Isosakuranetin	3.24	11.22	25.04	102.75	37.66	14.13	3.86
Luteolin-7-rutinoside	0.83	0.88	1.52	2.91	<LOD	<LOD	1.06
Myricetin	0.02	0.00	0.00	0.00	<LOD	<LOD	<LOD
Naringenin	1.62	1.43	9.94	4.43	14.33	28.23	5.16
Naringin	<LOD	0.80	<LOD	0.59	<LOD	<LOD	<LOD
Nepetin	0.03	<LOD	0.12	0.12	0.03	0.02	0.39
Nepetin-7 glucoside	0.12	<LOD	0.08	0.28	<LOD	<LOD	0.25
Quercetin	0.03	<LOD	<LOD	<LOD	<LOD	<LOD	0.06
Rhamnocitrin	<LOD	1.03	3.47	4.55	7.42	8.75	8.68
Rutin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.03
Verbascoside	4.48	<LOD	10.32	17.26	<LOD	<LOD	7.41
Oleanoic acid	0.94	<LOD	0.74	<LOD	0.92	1.15	1.95
Chlorogenic acid	0.16	0.54	1.51	50.27	<LOD	<LOD	0.51
Tormentic acid	1.13	<LOD	<LOD	<LOD	0.80	1.90	0.79
Diosmetin	18.13	54.82	65.54	81.91	42.78	38.85	55.04

The origins of propolis extracts are abbreviated with numbers as follows **1**: Bahceli; **2**: Karaoglanoglu; **3**: Girne; **4**: Tirmen; **5**: Guzelyurt; **6**: Karpaz; **7**: Taskent).

The validation parameters consisted of linearity, repeatability, recovery, limit of detection (LOD) and limit of quantification (LOQ) experiments. The linearity for each compound for the reported method was determined by analyzing the standard solution. The summary of validation and uncertainty data, the correlation coefficients (R^2) and linear regression equations of the reported compounds were presented in Table S1 (Supplementary material), where *y* is the peak area and *x* is the concentration. We reported detailed procedures of uncertainty evaluation in our previous reports (Sarikahya et al., 2019). Summarized validation and uncertainty data are presented in Table S1 (Supplementary material).

The secondary metabolite compositions of ethanol extracts of seven Cypriot propolis were identified by LC-HRMS, for the first time. Fifteen flavonoids [(+)-*trans* taxifolin, dihydrokaempferol, quercetin, apigenin, naringenin, acacetin, 3-*O*-methyl-quercetin, nepetin, rhamnocitrin, hispidulin, isosakuranetin, eupatilin, chrysin, myricetin, diosmetin], eight flavonoid glycosides [naringin, hyperoside, luteolin-7-*O*-rutinoside, rutin, hesperidin, apigenin-7-glucoside, nepetin-7-glucoside, quercetin-3-*O*-rutinoside], five phenolic acids [ellagic acid, caffeic acid, *p*-coumaric acid, *trans*-ferulic acid, chlorogenic acid], a phenylethanoid glycoside [verbascoside], three triterpenoids [hederagenin, oleanoic acid, tormentic acid], together with biosynthetically important and common dicarboxylic acid [fumaric acid] were identified and quantitated. The amount of those compounds in the seven ethanol-water extracts were measured simultaneously (Table 2). Among the detected compounds 25 compounds were identified in propolis

from Bahceli and Taskent, 20 compounds in propolis from Girne and Tirmen, 16 compounds in propolis from Karaoglanoglu, 14 compounds in propolis from Karpaz, and 13 compounds in propolis from Guzelyurt, and quantified based on their retention times and MS pattern in comparison with the data of references. According to the results of LC-HRMS analysis, while the major flavonoids found were isosakuranetin, naringenin, rhamnocitrin, diosmetin, chrysin and acacetin, verbascoside and chlorogenic acid were detected as major constituents in the water-ethanol extracts of propolis samples. Diosmetin content of all extracts was the most abundant at the level between 18.13 and 81.91 mg/g except the propolis from Tirmen. Isosakuranetin was found to be the major component in propolis from Tirmen (102.75 mg/g). Isosakuranetin was also detected in high amounts in the propolis samples from Karaoglanoglu, Girne, Guzelyurt and Karpaz (11.22–37.66 mg/g). The other flavonoid compound, naringenin, was also detected in notable amounts in propolis from Girne (9.94 mg/g), Guzelyurt (14.33 mg/g), and Karpaz (28.23 mg/g). The main phenolic acid was chlorogenic acid, which was present at the level of 50.27 mg/g in the water-ethanol extract of propolis from Tirmen. Verbascoside is a phenylethanoid glycoside that was detected in propolis from Girne, Tirmen, and Taskent with relatively high amounts (7.41–17.26 mg/g). Hederagenin, which is a triterpene compound, was only detected in the propolis from Bahceli at the level of 5.25 mg/g. The other triterpenes oleanoic and tormentic acids were identified in all of the propolis except in the propolis samples of Karaoglanoglu, Girne, and Tirmen. Accordingly, it is concluded that propolis samples from different locations in Cyprus have dissimilar chemical contents. The propolis from Tirmen was identified as the richest in terms of flavonoid and phenolic compounds, compared with the others, which may be one of the reasons for its higher cytotoxic and antioxidant activities (see below).

HPLC-DAD profiling of propolis samples and HCA analysis

HPLC chromatograms of the samples at 254 nm are shown in Figure S2 (Supplementary material). According to the HPLC chromatogram chemical profiles of the propolis samples are similar to each other. According to the results obtained from HCA it is possible to separate samples into two main groups. Members of the first group are propolis samples collected from Guzelyurt, Bahceli, Karpaz and Taskent (Supplementary material, Figure S3). The members of the second group are Karaoglanoglu, Girne and Tirmen (Supplementary material, Figure S4). When the similarities within the first group are examined samples from Karpaz and Taskent appear to be most similar to each other. Furthermore, the Bahceli sample is similar to these two more than the sample collected from Guzelyurt (Supplementary material, Figure S3). In the second group Karaoglanoglu and Tirmen samples appear to be more similar to each other than the Girne sample (Supplementary material, Figure S4).

Volatile composition of the propolis samples

In the current study, volatile components of propolis samples were characterized by HS-SPME coupled with GC-MS system. Volatile compositions of the propolis samples are given in Table 1, according to their relative retention indices and percentages. Eighty-three volatile components representing 90.3–97.8% of the total contents were identified in the propolis samples: 42 compounds in the propolis sample from Tirmen region, 48 compounds in the propolis sample from Karaoglanoglu, 42 compounds in the propolis sample from Girne, 41 compounds in the propolis sample Tirmen, 50 compounds in the propolis sample from Guzelyurt, 54 compounds in the propolis sample from Karpaz, and 45 compounds in the propolis sample from Taskent were characterized. α-Pinene (34.2–66.8%) was found as major component for all the propolis samples (34.2%–66.8%).

Cytotoxicity activity

Propolis extracts were tested for their cytotoxicity effect by MTT assay and all propolis extracts from different geographical origins showed significant cytotoxicity in all cell lines in a dose-dependent manner (Table 3). Vehicle effect (DMSO) was also tested against culture medium treatment (Supplementary material, Figure S5). Variations in IC_{50} values of different propolis extracts were observed between each other on different cell lines tested.

Nitric oxide analysis and antioxidant activity

The potential anti-inflammatory effect of extracts that inhibit the expression of NO was determined to evaluate the immunoregulatory activity. All extracts were tested for inhibition of NO production via LPS-stimulated RAW 264.7 cells for 24 h. iNOS IC_{50} values of propolis extracts from different geographical regions varied between 2.6 and 13.0 μg/mL (Table 4). All propolis extracts from different geographical regions exhibited DPPH scavenging activities ranging from 79.51% to 88.82% (Table 4).

Table 3. IC_{50} values of propolis extracts collected from different regions of Cyprus following 48 h exposure of cancerous and non-cancerous cells.

	IC_{50} (μg/mL)							
	Bahceli	Karaoglanoglu	Girne	Tirmen	Guzelyurt	Karpaz	Taskent	Doxorubicin
HEK293	12.92 ± 1.06	8.38 ± 0.63	11.94 ± 2.17	6.89 ± 0.67	13.03 ± 0.79	10.84 ± 0.89	8.97 ± 1.76	11.81 ± 2.64
SHSY5Y	14.90 ± 0.77	5.32 ± 0.01	9.76 ± 0.93	7.60 ± 1.75	3.29 ± 0.23	3.46 ± 0.29	6.11 ± 1.67	6.83 ± 1.14
PC3	16.34 ± 1.13	4.27 ± 0.15	11.98 ± 1.24	6.25 ± 1.63	25.73 ± 3.21	31.09 ± 1.83	9.14 ± 1.18	5.52 ± 2.53
PANC-1	16.71 ± 1.69	11.56 ± 1.27	12.97 ± 0.80	9.33 ± 0.16	13.91 ± 1.16	20.59 ± 2.36	16.21 ± 1.40	10.56 ± 3.21
MDA-MB-231	9.55 ± 0.03	3.54 ± 0.20	9.47 ± 0.60	3.25 ± 0.54	11.75 ± 0.89	16.48 ± 1.69	15.46 ± 3.20	11.68 ± 1.23
MCF-7	39.95 ± 8.82	7.68 ± 0.40	7.56 ± 0.48	6.16 ± 0.48	16.48 ± 1.52	27.46 ± 4.41	3.84 ± 0.28	17.81 ± 0.88
HeLa	2.49 ± 0.40	2.36 ± 0.38	0.76 ± 0.07	1.44 ± 0.27	13.46 ± 1.31	44.57 ± 6.73	16.79 ± 0.46	8.97 ± 0.69
A549	12.27 ± 0.34	9.97 ± 1.65	5.05 ± 0.73	4.73 ± 0.81	12.71 ± 3.43	16.58 ± 2.32	9.99 ± 1.56	4.19 ± 2.99
CaCo-2	3.43 ± 1.52	2.57 ± 1.05	7.38 ± 2.68	7.14 ± 2.20	3.33 ± 1.41	7.81 ± 0.79	23.25 ± 1.49	4.47 ± 0.23

HEK293, Human embryonic kidney cells; SHSY5Y, human neuroblastoma cells; PC3, human prostate cancer cells; PANC-1, human pancreatic cancer cells; MDA-MB-231, human triple negative breast cancer cells; MCF-7, human HER2+ breast cancer cells; HeLa, human cervical cancer cells; A549, human lung adenocarcinoma cells; CaCo-2, human colon carcinoma cells.

Table 4. IC_{50} values of inducible nitric oxide synthase (iNOS) inhibition in LPS-stimulated RAW 264.7 cells and DPPH scavenging activity (%) of Cypriot propolis extracts following 24 h exposure.

Samples	iNOS IC_{50} values (μg/mL)	DPPH scavenging activity (%)
Bahceli	5.45 ± 0.07	87.15 ± 0.10
Karaoglanoglu	13.0 ± 2.82	79.51 ± 0.10
Girne	12.4 ± 2.09	84.31 ± 0.79
Tirmen	11.0 ± 0.70	76.88 ± 0.29
Guzelyurt	3.6 ± 1.26	88.82 ± 0.10
Karpaz	2.6 ± 0.70	81.94 ± 0.39
Taskent	6.55 ± 0.78	85.28 ± 0.20
Doxorubicin	3.5 ± 0.56	–

RAW 264.7, murine macrophage cells.

Table 5. Minimum inhibitory concentration (MIC) values of Cypriot propolis extracts for microorganisms following 24 h exposure (μg/mL).

	MIC values (μg/mL) for the samples and reference antimicrobial agents								
Microorganisms	1	2	3	4	5	6	7	Gent.	FC
E. coli O157-H7	125	125	250	125	62.5	250	125	15.6	–
S. aureus ATCC 25923	62.5	31.2	62.5	125	31.2	62.5	31.2	7.8	–
S. epidermidis ATCC 12228	62.5	125	125	125	31.2	62.5	31.2	7.8	–
E. faecium DSM 13590	62.5	62.5	31.2	31.2	31.2	31.2	31.2	15.6	–
E. faecalis ATCC 29212	62.5	62.5	62.5	62.5	31.2	31.2	62.5	15.6	–
S. Typhimurium CCM 5445	125	125	125	250	62.5	125	125	7.8	–
L. monocytogenes ATCC 19115	62.5	31.2	62.5	62.5	62.5	62.5	31.2	3.9	–
P. aeroginosa ATCC 27853	125	125	125	250	62.5	125	125	15.6	–
C. tropicalis RSKK 2412	62.5	31.2	125	62.5	62.5	62.5	125	–	15.6
C. albicans ATCC 10231	125	125	125	125	62.5	62.5	125	–	15.6

The origin of propolis extracts are abbreviated with numbers as follows **1**: Bahceli; **2**: Karaoglanoglu**;** **3**: Girne; **4**: Tirmen; **5**: Guzelyurt; **6**: Karpaz; **7**: Taskent. Gent: Gentamicin; FC: Flucytosine.

Antimicrobial activity

The antimicrobial activities of the propolis extracts were tested against reference bacterial and yeast strains. The MIC values for the propolis samples are shown in Table 5 while those in Table S2 (Supplementary material) demonstrate the MBC and MFC values. All propolis extracts evaluated in this study showed antimicrobial effect against Gram-positive and Gram-negative bacteria as well as against yeasts with MIC ranging from 31.2 μg/mL to 250 μg/mL (Table 5).

Discussion

Propolis is one of the candidates for natural medical products with its enormous cytotoxic, immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial activities (Silva-Carvalho et al., 2014). The biological activities of propolis come from its secondary metabolite ingredients for instance terpenes, pterocarpans, prenylated benzophenones, and especially phenolic compounds (Rufatto et al., 2017). It is well known that propolis is highly variable in terms of compounds and biological activities due to the variations in the plant flora and season of collection (Wagh, 2013). In this study, seven different propolis samples from seven geographical regions in the northern parts of Cyprus were collected. This study suggests the first publication of Cypriot propolis in terms of biological activities and chemical compositions in the literature.

Propolis extracts can be prepared by using various methods such as traditional maceration extraction, microwave-assisted extraction, and ultrasound extraction. Propolis extracts of Cyprus were prepared

via water-ethanol (70%) mixture by using ultrasound extraction technique that was considered as the best extraction method for propolis (Trusheva et al., 2007).

The secondary metabolite compositions were identified by LC-HRMS, for the first time (Table 2). The High-Resolution Mass Spectrometry and MS/MS (parent/daughter ions patterns) methods were used at the same time, in addition to the retention time of the LC. Our LC-HRMS database identified compounds including phenolics, coumarins, flavonoids, iridoids, alkaloids, diterpenoids, triterpenoids, and saponins. The bees collect samples from many plants (not only from the flowers, secretions of the trees, stems and leaves), and carry everything to their hive. A single plant can synthesize more than 1000 secondary metabolites and from the whole flora, these numbers can be counted as several hundred thousand or more. As mentioned before in the introduction section, caffeic acid phenethyl ester (CAPE) is a major constituent of moderate propolis. In tropical region propolis, especially Brazilian green propolis, the leading chemical phenolics are caffeic acid, cinnamic acid, *p*-coumaric acid, and ferulic acid (Ishida et al., 2018). In contrast, although Cyprus lies within the Mediterranean, the major compounds were found to be verbascoside and chlorogenic acid. This is an unexpected result that can be attributed to the unique climate, geographic diversity of Cyprus Island and to the several *Verbascum* species, which are the main sources of verbascoside, growing in the natural environment of Cyprus (Fadel et al., 2020; Hanoğlu et al., 2019). MS fingerprints, MS/MS patterns, and RT of the standard is in agreement with the compound. This study does not report only the identification of the compounds but also reports the analytical method validation aspects and properties of the compounds including strong analytical and metrological discussion in it. Thus, a simple rule of analytical chemistry is the selectivity experiments of the target compounds according to EURACHEM CITAC guide and Pharmacopeia. Thus, all of the data of method validation clearly shows that verbascoside, a phenylethanoid glycoside, was determined in the propolis samples correctly. And, regarding the numbers of identified and quantified compounds by LC-HRMS, these kinds of reports are limited in the literature.

Volatile compounds are one of the most common secondary metabolites in plants, and play a complex, vital role in relationships between plants and their ecological environments (Rufatto et al., 2017; Silva-Carvalho et al., 2014). Although volatile compounds are found in low concentrations in propolis, their biological activities make them significant for propolis characterization (Bankova et al., 2014). In this study, HS-SPME-GC/MS was carried out successfully for the analysis of volatile compounds of propolis samples. α-Pinene was found as a major component in the samples of propolis collected from Southern Italy, Greece, Brazil, Mexico (Yucatan), Estonia and Uruguay (Ioshida et al., 2010; Kaškonienė et al., 2014; Melliou et al., 2007; Simionatto et al., 2012). However, the α-pinene amount of 64 propolis samples from Hakkari, Turkey were reported in lower levels (Bayram et al., 2018), and chemical composition is different from our data. Variation of the volatile chemical composition of Cyprus propolis samples could be caused due to different environmental factors that alter the production/concentration of secondary metabolites in botanical sources.

According to the Cypriot propolis biological activity results, extracts from various geographical regions demonstrated different levels of cytotoxicty, antioxidant, antimicrobial and iNOS inhibition effects. It has been reported by Huang and colleagues (Huang et al., 2014) that until 2000, more than 300 different molecules were identified as components of propolis from various natural products classes like flavonoids, phenolics, terpenes, essential oils, etc. The phenolic and flavonoid compounds are correlated with the antioxidant and antitumor activity of propolis. Cypriot propolis extracts especially Karaoglanoglu and Tirmen samples showed remarkable cytotoxicity on PC-3 and PANC-1 cells which can be attributed to their rich chemical content in terms of flavonoid and phenolic compounds. If we elaborate on the major flavonoids, we can see that diosmetin and isosakuranetin were detected in high amounts in these propolis samples. In particular, the highest inhibition on cytotoxicity of Tirmen propolis was shown against A549 cells, which may be related to the content of isosacuranetin (102.75 mg/g), diosmetin (81.91 mg/g) and chlorogenic acid (50.27 mg/g). The propolis from Guzelyurt region showed the highest antioxidant activity since it contains most various flavanoid compounds in line with the literature findings (Ahmed et al., 2017; Kumar et al., 2008). Based on our HCA results propolis samples from Karaoglanoglu and Tirmen are similar to each other. Parallel to these results those samples showed significant inhibition on cancer cell lines and decadent performance in DPPH assays. In addition, according to the volatile composition data, Karaoglanoglu and Tirmen were found to be rich in limonene constituents. This might hint toward the responsible constituent for significant cytotoxicity. These two regions are placed on the edges of Besparmak Mountains that are in the countryside regions providing a similar climate and more natural flora. Although samples from Karpaz and Taskent appear to be very similar to each other they did not show the same biological activity (Tables 3–5). For example, the cytotoxic activities of these samples on the PC3 cell line are

different than each other. These results would indicate different activity mechanisms of the cell lines or different types of synergistic effects of components in the samples.

It is known that propolis extracts have immunomodulatory activities to inhibit NO production due to their flavonoid content (Olszanecki et al., 2002). NO, a bioactive molecule produced by inducible nitric oxide synthase (iNOS), has concentration-dependent pro- or antitumor effects in many cancer types including breast, lung, colon, etc. INOS is known for its pro-inflammatory mediator activity by binding to calmodulin and producing NO as an immune defence mechanism (Vannini et al., 2015). The tested propolis extracts have been found to exhibit high iNOS inhibition activity potential. Karpaz sample showed the highest activity on iNOS inhibition with IC_{50} value of 2.60 μg/mL. It also demonstrated a more significant effect on iNOS inhibition than the positive control doxorubicin (IC_{50}: 3.5 μg/mL). Compared with the literature, it is observed that Cyprus propolis samples have strong iNOS inhibition capacity (Blonska et al., 2004; Paulino et al., 2006; Song et al., 2002). This might be due to the rich phenolic and flavonoid content of Cypriot propolis extracts, which are directly related to the climate and the flora of the collection point. The data obtained from the DPPH study demonstrated that the antioxidant activity of propolis extracts from Cyprus had slight variations between each other. Eventually, the Guzelyurt propolis sample has the highest antioxidant activity among the six other propolis. Furthermore, DPPH radical scavenging activities of propolis ethanol extracts show similar results compared to the antioxidant activities of propolis ethanol extract from different geographical origins (Argentina, Australia, Brazil, Bulgaria, Chile, China, Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States, and Uzbekistan) (Kumazawa et al., 2004).

Propolis is also known for its antimicrobial activities which are related to their high flavonoid contents (Daikh et al., 2020; Drago et al., 2000). The antibacterial effects of Cypriot propolis samples represented variations based on differences among bacterial strains and propolis samples used. Even though all of the tested Gram-positive and Gram-negative bacterial strains tested were sensitive to all propolis samples, the MIC values of Gram-negative bacteria were higher than that of Gram-positive bacteria. While MIC values of Gram-positive microorganisms ranged from 31.2 to 125 μg/mL, bacteriostatic effect has been demonstrated against Gram-negative microorganisms when their MIC is between 62.5 and 250 μg/mL. Variations on inhibitory effects can be attributed to the differences in cell wall and membrane structure of the corresponding organisms. Drago et al. (2000) also observed that low propolis concentrations revealed the presence of a bacteriostatic effect rather than bactericidal activity. Propolis sample from Guzelyurt region exhibited the highest antibacterial activity against *S. aureus, S. epidermidis, E. faecium* and *E. faecalis* with MIC values of 31.2 μg/mL. All propolis extracts also showed antifungal activity against *Candida* strains tested corresponding to the data in the literatures (Daikh et al., 2020; Drago et al., 2000; Salomao et al., 2004). Karaoglanoglu sample exhibited the highest antifungal activity among other samples tested. However, propolis sample from Guzelyurt demonstrated antimicrobial activity at lower concentrations and it also showed the highest antifungal activity against *C. tropicalis* when compared to other propolis extracts. It may also be suggested that the propolis sample in Guzelyurt to show the strongest antmicrobial activity may be due to having the highest antioxidant activity. It is also suggested that the presence of phenolic compounds in the propolis may be responsible for antimicrobial activity by causing cytoplasmic content leakage through altering cell surface charge and the hydrophobicity of Gram-positive and Gram-negative bacteria on the cell membrane (Borges et al., 2013).

Conclusions

In conclusion, this is the first study in the literature covers the investigation of chemical composition and biological activities of Cypriot propolis samples. Interestingly, although propolis samples from different geographical regions are composed of mainly caffeic acid, cinnamic acid, *p*-coumaric acid, and ferulic acid, major secondary metabolites of the evaluated Cypriot propolis samples have been identified as verbascoside and chlorogenic acid. Besides that, nonoxygnetaed monoterpene, α-pinene, was found to be major monoterpene in all propolis samples, reported herein.

In this study, we observed that propolis extracts from different locations in the northern part of Cyprus exerted strong cytotoxicity against cancerous cell lines, as well as promising antimicrobial and antioxidant activities. Among the extracts from various regions of Cyprus, propolis collected from the Karaoglanoglu region exerted a superior cytotoxic effect on cancer cells, followed by Tirmen from the eastern part of the island. These two regions also conferred a similar profile in the LC-HRMS study, as both of the regions are within the Besparmak Mountains, having similar climates and natural flora. The Guzelyurt sample demonstrated the highest antimicrobial and antioxidant activities when compared to other tested samples. The Karpaz and Guzelyurt samples, collected from a dry climate with plain plateau, were also investigated as the most potent iNOS inhibitors according to the study. Data obtained from the study altogether suggests that

propolis extracts from Cyprus could be promising candidates as a natural nutrition supplement due to their superior biological activities.

Acknowledgements

We would like to thank Kırata Kasapoğlu, President of the Northern Cyprus Beekeepers Foundation for providing us with 6 propolis samples from various geographical regions of Cyprus. We would like to thank Hülya-Soner Ortan for providing us a sample of Taşkent propolis. We would like to thank Sami Gürsel Özverel for mediating the connection with the Northern Cyprus Beekeepers Foundation for propolis samples. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Ayşe Nalbantsoy http://orcid.org/0000-0002-3852-1974
Nazli Boke Sarıkahya http://orcid.org/0000-0002-8200-8561
Cenk Serhan Özverel http://orcid.org/0000-0001-9932-4774
Betül Demirci http://orcid.org/0000-0003-2343-746X
Ahmet C. Goren http://orcid.org/0000-0002-5470-130X

References

Ahmed, R., Tanvir, E. M., Hossen, S., Afroz, R., Ahmmed, I., Rumpa, N.-E.-N., Paul, S., Gan, S. H., Sulaiman, S. A., & Khalil, I. (2017). Antioxidant properties and cardioprotective mechanism of Malaysian propolis in rats. *Evidence-Based Complementary and Alternative Medicine*, *2017*, 5370545.

Bankova, V., Popova, M., & Trusheva, B. (2014). Propolis volatile compounds: Chemical diversity and biological activity: A review. *Chemistry Central Journal*, *8*(1), 28.

Bankova, V. S., De Castro, S. L., & Marcucci, M. C. (2000). Propolis: Recent advances in chemistry and plant origin. *Apidologie*, *31*(1), 3–15. https://doi.org/10.1051/apido:2000102

Bayram, N. E., Sorkun, K., Öz, G. C., Salih, B., & Topçu, G. (2018). Chemical characterization of 64 propolis samples from Hakkari. *Records of Natural Products*, *12*(6), 569–581. https://doi.org/10.25135/rnp.78.16.12.585

Benguedouar, L., Boussenane, H. N., Wided, K., Alyane, M., Rouibah, H., & Lahouel, M. (2008). Efficiency of propolis extract against mitochondrial stress induced by antineoplastic agents (doxorubicin and vinblastin) in rats. *Indian Journal of Experimental Biology*, *46*(2), 112–119.

Blonska, M., Bronikowska, J., Pietsz, G., Czuba, Z. P., Scheller, S., & Krol, W. (2004). Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774 a.1 macrophages. *Journal of Ethnopharmacology*, *91*(1), 25–30. https://doi.org/10.1016/j.jep.2003.11.011

Borges, A., Ferreira, C., Saavedra, M. J., & Simoes, M. (2013). Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial Drug Resistance*, *19*(4), 256–265. https://doi.org/10.1089/mdr.2012.0244

Bueno-Silva, B., Kawamoto, D., Ando-Suguimoto, E. S., Casarin, R. C. V., Alencar, S. M., Rosalen, P. L., & Mayer, M. P. A. (2017). Brazilian red propolis effects on peritoneal macrophage activity: Nitric oxide, cell viability, pro-inflammatory cytokines and gene expression. *Journal of Ethnopharmacology*, *207*, 100–107. https://doi.org/10.1016/j.jep.2017.06.015

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology*, *36*(4), 347–363. https://doi.org/10.1016/S0278-6915(97)00145-2

Castaldo, S., & Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia*, *73 Suppl 1*(1), S1–S6.

Chapman, F. E. (1980). Cyprus trees and shrubs, Cyprus Goverment's Copyright Reserved, Nicosia.

Chu, Y.-H., Chang, C.-L., & Hsu, H. F. (2000). Flavonoid content of several vegetables and their anti-oxidant activity. *Journal of the Science of Food and Agriculture*, *80*(5), 561–566. https://doi.org/10.1002/(SICI)1097-0010(200004)80:5<561::AID-JSFA574>3.0.CO;2-#

CLSI. (2009). *Performance standards for antimicrobial susceptibility testing, nineteenth informational supplement* [Approved Standard M100-S19]. Clinical Laboratory Standards Institute.

Daikh, A., Segueni, N., Dogan, N. M., Arslan, S., Mutlu, D., Kivrak, I., Akkal, S., & Rhouati, S. (2020). Comparative study of antibiofilm, cytotoxic activity and chemical composition of Algerian propolis. *Journal of Apicultural Research*, *59*(2), 160–169. https://doi.org/10.1080/00218839.2019.1701777

Dagli, M., Sarikahya, N. B., Nalbantsoy, A., & Kirmizigul, S. (2019). Comparative phytochemical screening and cytotoxic efficacy of endemic *Cephalaria tuteliana*. *Natural Product Communications*, *14*(10), 1–5.

De Vecchi, E., & Drago, L. (2007). Propolis' antimicrobial activity: What's new? *Le Infezioni in Medicina*, *15*(1), 7–15.

Drago, L., Mombelli, B., De Vecchi, E., Fassina, M. C., Tocalli, L., & Gismondo, M. R. (2000). *In vitro* antimicrobial activity of propolis dry extract. *Journal of Chemotherapy*, *12*(5), 390–395.

Fadel, S. R., Bendif, H., Guedes, L., Andre, R., Pacheco, R., Guedes, R., Merabti, K., Miara, M. D., & Serralheiro, M. L. (2020). Bioactivities of iridoids and flavonoids present in decoctions from aerial parts of *Verbascum betonicifolium*. *European Journal of Integrative Medicine*, *37*, 101171. https://doi.org/10.1016/j.eujim.2020.101171

Hamad, H. O., Alma, M. H., Gulcin, I., Yilmaz, M. A., & Karaogul, E. (2017). Evaluation of phenolic contents and bioactivity of root and nutgall extracts from Iraqian *Quercus infectoria* Olivier. *Records of Natural Products*, *11*, 205–210.

Hanoğlu, D. Y., Hanoğlu, A., Yusufoğlu, H., Demirci, B., Başer, K. H. C., Çalı ş, İ., & Özkum Yavuz, D. (2019). Phytochemical investigation of endemic *Sideritis cypria* post. *Records of Natural Products*, *14*(2), 105–115. https://doi.org/10.25135/rnp.140.18.11.1079

Hochmuth, D. H. (2008). *MassFinder 4.0*. Hochmuth Scientific Consulting.

Huang, S., Zhang, C.-P., Wang, K., Li, G. Q., & Hu, F.-L. (2014). Recent advances in the chemical composition of propolis. *Molecules (Basel, Switzerland)*, *19*(12), 19610–19632. https://doi.org/10.3390/molecules191219610

Ilseven, S. (2017). Analysis of maquis and garrigue communities on the island of Cyprus and comparison with calabrian pine communities in terms of ecological characteristics. *Journal of Environmental Biology*, *38*, 955–960.

Ioshida, M. D. M., Young, M. C. M., & Lago, J. H. G. (2010). Chemical composition and antifungal activity of essential oil from Brazilian propolis. *Journal of Essential Oil Bearing Plants*, *13*(5), 633–637. https://doi.org/10.1080/0972060X.2010.10643873

Ishida, Y., Gao, R., Shah, N., Bhargava, P., Furune, T., Kaul, S. C., Terao, K., & Wadhwa, R. (2018). Anticancer activity of honeybee propolis: functional insights to the role of caffeic acid phenethyl ester and its complex with γ-cyclodextrin. *Integrative Cancer Therapies*, *17*(3), 867–873.

Kaškonienė, V., Kaškonas, P., Maruška, A., & Kubilienė, L. (2014). Chemometric analysis of volatiles of propolis from different regions using static headspace GC-MS. *Central European Journal of Chemistry*, *12*(6), 736–746.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., & Popov, S. (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *Journal of Ethnopharmacology*, *64*(3), 235–240.

Kumar, N., Mueen Ahmad, K. K., Dang, R., & Husain, A. (2008). Antioxidant and antimicrobial activity of propolis from Tamil Nadu zone. *Journal of Medicinal Plants Research*, *2*(12), 361–364.

Kumazawa, S., Hamasaka, T., & Nakayama, T. (2004). Antioxidant activity of propolis of various geographic origins. *Food Chemistry*, *84*(3), 329–339. https://doi.org/10.1016/S0308-8146(03)00216-4

McLafferty, F. W., & Stauffer, D. B. (1989). *The Wiley/NBS registry of mass spectral data*. J Wiley and Sons.

Meikle, R. D. (1985). *Flora of Cyprus*. Bentham and Moxon Trust Press.

Melliou, E., Stratis, E., & Chinou, I. (2007). Volatile constituents of propolis from various regions of Greece–antimicrobial activity. *Food Chemistry*, *103*(2), 375–380. https://doi.org/10.1016/j.foodchem.2006.07.033

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, 55–63.

Nguyen, S. T., Nguyen, H. T., & Truong, K. D. (2020). Comparative cytotoxic effects of methanol, ethanol and DMSO on human cancer cell lines. *Biomedical Research and Therapy*, *7*(7), 3855–3859. https://doi.org/10.15419/bmrat.v7i7.614

Olszanecki, R., Gebska, A., Kozlovski, V. I., & Gryglewski, R. J. (2002). Flavonoids and nitric oxide synthase. *Journal of Physiology and Pharmacology*, *53*(4 Pt 1), 571–584.

Onbas, R., Kazan, A., Nalbantsoy, A., & Yesil-Celiktas, O. (2016). Cytotoxic and nitric oxide inhibition activities of propolis extract along with microencapsulation by complex coacervation. *Plant Foods for Human Nutrition*, *71*(3), 286–293.

Park, Y. K., Alencar, S. M., & Aguiar, C. L. (2002). Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry*, *50*(9), 2502–2506. https://doi.org/10.1021/jf011432b

Paulino, N., Teixeira, C., Martins, R., Scremin, A., Dirsch, V. M., Vollmar, A. M., Abreu, S. R. L., de Castro, S. L., & Marcucci, M. C. (2006). Evaluation of the analgesic and anti-inflammatory effects of a Brazillian green propolis. *Planta Medica*, *72*(10), 899–906. https://doi.org/10.1055/s-2006-947185

Quang, d. N., Harinantenaina, L., Nishizawa, T., Hashimoto, T., Kohchi, C., Soma, G., & Asakawa, Y. (2006). Inhibition of nitric oxide production in RAW 264.7 cells by azaphilones from xylariaceous fungi. *Biological & Pharmaceutical Bulletin*, *29*(1), 34–37.

Rufatto, L. C., Amilton dos Santos, D., Marinho, F., Henriques, J. A. P., Ely, M. R., & Moura, S. (2017). Red propolis: Chemical composition and pharmacological activity. *Asian Pacific Journal of Tropical Biomedicine*, *7*(7), 591–598. https://doi.org/10.1016/j.apjtb.2017.06.009

Salomao, K., Dantas, A. P., Borba, C. M., Campos, L. C., Machado, D. G., Aquino, N. F. R., & de Castro, S. L. (2004). Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis. *Letters in Applied Microbiology*, *38*(2), 87–92.

Sarikahya, N. B., Goren, A. C., & Kirmizigul, S. (2019). Simultaneous determination of several flavonoids and phenolic compounds in nineteen different *Cephalaria* species by HPLC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, *173*, 120–125.

Silva-Carvalho, R., Miranda-Gonçalves, V., Ferreira, A. M., Cardoso, S. M., Sobral, A. J. F. N., Almeida-Aguiar, C., & Baltazar, F. (2014). Antitumoural and antiangiogenic activity of Portuguese propolis in *in vitro* and *in vivo* models. *Journal of Functional Foods*, *11*, 160–171. https://doi.org/10.1016/j.jff.2014.09.009

Simionatto, E., Facco, J. T., Morel, A. F., Giacomelli, S. R., & Linares, C. E. (2012). Chiral analysis of monoterpenes in volatile oils from propolis. *Journal of the Chilean Chemical Society*, *57*(3), 1240–1243. https://doi.org/10.4067/S0717-97072012000300009

Song, Y. S., Park, E.-H., Hur, G. M., Ryu, Y. S., Kim, Y. M., & Jin, C. (2002). Ethanol extract of propolis inhibits nitric oxide synthase gene expression and enzyme activity. *Journal of Ethnopharmacology*, *80*(2-3), 155–161.

Tasdemir, D., Demirci, B., Demirci, F., Dönmez, A. A., Baser, K. H. C., & Rüedia, P. (2003). Analysis of the volatile components of five Turkish rhododendron species by headspace solid-phase microextraction and GC-MS (HS-SPME-GC-MS). *Zeitschrift für Naturforschung C*, *58*(11-12), 797–803. https://doi.org/10.1515/znc-2003-11-1208

Tiveron, A. P., Rosalen, P. L., Franchin, M., Lacerda, R. C., Bueno-Silva, B., Benso, B., Denny, C., Ikegaki, M., & Alencar, S. M. (2016). Chemical characterization and antioxidant, antimicrobial, and anti-inflammatory activities of south Brazilian organic propolis. *PLoS One*, *11*(11), e0165588-18.

Trusheva, B., Trunkova, D., & Bankova, V. (2007). Different extraction methods of biologically active components from propolis: A preliminary study. *Chemistry Central Journal*, *1*, 13.

Vannini, F., Kashfi, K., & Nath, N. (2015). The dual role of iNOS in cancer. *Redox Biology*, *6*, 334–343.

Wagh, V. D. (2013). Propolis: A wonder bees product and its pharmacological potentials. *Advances in Pharmacological Sciences*, *2013*, 308249. https://doi.org/10.1155/2013/308249

Yan-Hwa, C., Chang, C.-L., & Hsu, H.-F. (2000). Flavonoid content of several vegetables and their antioxidant activity. *Journal of Science and Food Agriculture*, *80*(5), 561–566. https://doi.org/10.1002/(SICI)1097-0010(200004)