Food Chemistry 116 (2009) 452–461

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus

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ARTICLE INFO

Article history:
Received 17 December 2008
Received in revised form 23 January 2009
Accepted 19 February 2009

Keywords:
Propolis
Flavonoids
Terpenes
Anthraquinones
Antioxidant activity
Antimicrobial activity
Nisin

ABSTRACT

Chemical composition, antioxidant activity and *in vitro* antimicrobial activity of twelve propolis ethanolic extracts (PEE) from mainland Greece, Greek islands, and east Cyprus were determined. The PEE studied contained significant amounts of terpenes and/or flavonoids, anthraquinones – mainly emodin and chrysophanol – and low amounts of phenolic acids and their esters, presenting differences from typical European propolis, and similarities to East Mediterranean propolis. Simple polyphenols and terpenic acids content ranged between 11.9–373.5 and 7.23–286.5 mg/g of PEE, respectively, with anthraquinones representing the 1.3–28.9% of simple polyphenols. Despite differences in composition, the PEE samples exhibited significant antioxidant, antibacterial, and antifungal activities, affecting a wider spectrum of microorganisms than the food grade antibacterial nisin, and presenting lower or no activity against several *Lactobacillus* strains. The presence of significant amounts of terpenoids seemed to enhance the antimicrobial activity of PEE. The conclusion, given the non-toxic and natural origin of PEE, is that, besides their potential pharmaceutical and nutraceutical value, propolis balsams from Greece and Cyprus are attractive candidates for use as natural antioxidant and microbicidal additives in food systems, especially those containing lactic acid bacteria.

1. Introduction

Propolis is a resinous, strongly adhesive natural substance, collected by honeybees (*Apis mellifera* L.) from buds and leaves of trees and plants, mixed with pollen as well as enzymes secreted by bees (Marcucci, 1995). Bees use it as a general-purpose sealer, to smooth out the internal walls of the hive and as protective barrier against intruders (Burdock, 1998).

In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris (Burdock, 1998). Wax and organic debris are removed during processing, usually by ethanolic extraction, and the propolis tincture (balsam) thus obtained, contains the bulk of propolis bioactive constituents. More than 300 compounds, among which polyphenols, terpenoids, steroids, sugars and amino acids have been detected in raw propolis. Their abundance is influenced by botanical and geographical factors, as well as by the collection season (Ahn et al., 2007; Bankova, De Castro, & Marcucci, 2000; Kujumgiev et al., 1999).

Propolis is considered responsible for the low incidence of bacteria and moulds within the hive. The action against microorganisms is an essential characteristic of propolis, and humans have used it for centuries for its pharmaceutical properties (Bankova et al., 2000; Ghisalberti, 1978). Besides its antibacterial, antifungal and antiviral properties, propolis presents many other beneficial biological activities such as antioxidant, antiinflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory, antimutagenic, etc. (Banskota, Tezuka, & Kadota, 2001; Burdock, 1998; Kim, Lee, Aum, & Kim, 2008; Kujumgiev et al., 1999). For these reasons propolis has been used as a popular remedy in folk medicine, in apitherapy, as a constituent of biocosmetics, health foods and in numerous other purposes (Bankova et al., 2000; Banskota et al., 2001; Ghisalberti, 1978). Although reports of allergic reactions are not uncommon, propolis is relatively non-toxic, with a non-observed effect level (NOEL) of 1400 mg/kg body weight/day in a mouse study (Burdock, 1998).

Propolis antioxidant, antibacterial and antifungal properties, combined with the fact that several of its constituents are present in food and/or food additives, and are recognised as Generally Recognised as Safe (GRAS) (Burdock, 1998), make it an attractive candidate as a natural preservative in new food applications. This

Abbreviations: BSTFA, *bis*-(trimethylsilyl)-trifluoroacetamide; DPPH·, 1,1-diphenyl-2-picrylhydrazyl radical; FRAP, ferric reducing antioxidant potential; MIC, minimum inhibitory concentration; PEE, propolis ethanolic extract; TIC, total ion current; TMS, trimethylsilyl ether.

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doi:10.1016/j.foodchem.2009.02.060

meets the demand for natural antioxidants and antimicrobials, fuelled by the increasing consumer awareness for natural, minimally processed foods with traditional preservatives absent or at very low concentrations (Han & Park, 1995; Tosi, Ré, Ortega, & Cazzoli, 2007).

Due to geomorphological characteristics, the Greek flora presents high biodiversity with many endemic plants (Melliou & Chinou, 2004), something that is also true for Greek islands and Cyprus, as a result of the islands' isolation in relation to continental lands. This is expected to differentiate the composition of Greek and Cypriot propolis from that of typical European ones.

In literature, scarce data can be found about the composition, antimicrobial and antioxidant activity of Greek propolis extracts, and no data at all about propolis from Cyprus. The chemical composition and antimicrobial activity of one propolis extract and of the volatiles from five propolis obtained from Greek mainland and one Greek island have been reported by Melliou and Chinou (2004) and Melliou, Stratis, and Chinou (2007), respectively, while Velikova et al. (2000) have studied the composition of propolis from Greece, Bulgaria, Turkey and Algeria.

In the present study, we are reporting the chemical composition – including quantitative data for several simple polyphenols and terpenic acids – the antioxidant activity, and the antimicrobial properties of twelve propolis samples collected from 10 localities of mainland Greece, Greek islands and Cyprus. Moreover, we compare the inhibitory spectra of propolis samples with that of nisin, a GRAS antibacterial peptide used for several years in foods.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and chemicals

Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), analytical grade ethanol, quercetin, 3-(4-hydroxyphenyl)-1-propanol, homovanillic acid, phloretic acid, oleanolic acid, cinnamic acid, Folin-Ciocalteu reagent, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\cdot}$) were obtained from Aldrich (Steinheim, Germany). Tyrosol, protocatechuic acid, sinapic acid, *o*-coumaric acid, 3,4-dihydroxy-phenylacetic acid, and caffeic acid, were purchased from Fluka (Steinheim, Germany); ascorbic acid, 2,4,6-*tris* (2-pyridyl)-s-triazine (TPTZ), *p*-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, ursolic acid, vanillin, *p*-coumaric acid, chlorogenic acid, catechin, syringic acid, gallic acid, resveratrol, and ferulic acid were obtained from Sigma (Steinheim, Germany); vanillic acid was obtained from Serva (Heidelberg, Germany), pinocembrin, kaempferol, chrysin, naringenin, acacetin and apigenine from Extrasynthése (Genay-Sedex, France), myricetin and epicatechin from Fluka Biochemika (Steinheim, Germany), genistein from Alfa Aesar (Karlsruhe, Germany), and abietic acid from MP Biomedicals LLC (Irvine, CA). Nisin powder (1 g = 40×10^6 IU) was purchased from Sigma Chemicals (St. Louis, USA).

2.1.2. Bacterial strains and culture preparations

In this study, 18 pathogenic and non-pathogenic bacterial strains (target strains) and two pathogenic fungi were used. *Shigella dysenteriae* NCTC 2966, *Salmonella typhimurium* NCTC 12023, *Enterobacter aerogenes* NCTC 10006, *Yersinia enterocolitica* NCTC 10460, *Escherichia coli* NCTC 09001, *Staphylococcus aureus* NCTC 6571 (I), *S. aureus* ATCC 25923 (II), *Staphylococcus epidermidis* NCTC 11047, *Bacillus cereus* NCTC 7464 (I), *B. cereus* ATCC 9139 (II), *Listeria monocytogenes* NCTC 10357 (I), *L. monocytogenes* ATCC 7644 (II) and the yeasts *Candida tropicalis* ATCC 13801 and *Candida albicans* ATCC 10231 were provided by Agrolab S.A. (Agrolab S.A., Athens, Greece). *Lactobacillus bulgaricus* ACA-DC 101 was provided by the ACA-DC collection (Laboratory of Dairy Research, Agricultural University of Athens, Greece) while *Lactobacillus fermentum* F 12, *Lactobacilus casei* LC 14, *Lactobacillus delbrueckii* sub. *delbrueckii* LDD-C1, *Lactobacillus plantarum* LP 101, and *Lactobacillus helveticus* LH 09 were provided by Laboratory of Microbiology, Harokopio University, Athens, Greece. Lactic acid bacteria working cultures were maintained on Man Rogosa and Sharpe (MRS, Merck, Darmstand, Germany) agar slants while the remaining strains were maintained on Tryptone Soy agar slants (TSA, Merck) supplemented with 0.6% yeast extract (Sigma, St. Louis, MO, USA), (TSYEA). Yeasts were maintained on yeast extract-peptone-dextrose (YPD) agar slants. All slants were stored at 4 °C, and sub-cultured twice per month.

2.2. Methods

2.2.1. Samples collection and propolis extract preparation

Propolis samples were obtained from several locations of central and southern Greece, Aegean Sea islands, and Cyprus (Larnaka, SE Cyprus), as indicated in Fig. 1 and Table 1. Samples were collected during spring–summer of 2007; in two cases – KAR-I and LAR-I – samples collected during 2006 were obtained. Voucher specimens are deposited in the Laboratory of Chemistry – Biochemistry – Physical Chemistry of Foods, Department of Nutrition-Dietetics, Harokopio University, Athens, Greece. Crude propolis samples were frozen (−20 °C), grounded in a chilled grinder and small amounts (10 g) of pulverised crude propolis were extracted under stirring with a 10-fold volume of 70% ethanol solution in tightly closed bottles, for 3 days. Extraction was carried out at ambient temperature in the dark. To remove waxes and less soluble substances, the suspensions were subsequently frozen at −20 °C for 24 h, then filtered with Whatman No. 1 filter paper. The freezing-filtration cycle was repeated three times. The final filtrates represent the balsam (tincture) of propolis and are referred to as PEE (propolis ethanolic extract). The solutions were evaporated to near dryness on a rotary evaporator under reduced pressure at 40 °C, and then freeze-dried. The resulting powders were dissolved in 80:20 ethanol:water in order to get 5% w/v PEE stock solutions. The ethanolic extraction yields were determined gravimetrically in aliquots of the extracts and found to range from 23.9% to 61.2% of raw propolis samples (Table 1).

2.2.2. Derivatisation and GC–MS analysis of propolis extracts

PEE constituents were determined by GC–MS operating either in SCAN (total ion current, TIC) or selective ion monitoring (SIM) mode (Table 2). Samples were derivatised prior to analysis. For this purpose, a proper volume of PEE, containing no more than 1 mg of dry extract, was transferred into GC vials. The internal standard was added – 50 μl of 3-(4-hydroxyphenyl)-1-propanol solution (19.2 μg/ml) – the sample was evaporated to dryness under nitrogen, and derivatised by the addition of 250 μl BSTFA at 70 °C for 20 min. An aliquot (1 μl) of the derivatised sample was injected into the gas chromatograph at a split ratio 1:20. An Agilent (Wallborn, Germany) HP series GC 6890 N coupled with a HP 5973 MS detector, split – splitless injector and an HP 7683 autosampler were employed. Mass selective (MS) detector operated under electron impact ionisation (70 eV) and MS scan range was 50–800 Da. Analysis of the samples was achieved using an HP-5 MS capillary column (5% phenyl – 95% methylsiloxane, 30 m × 0.25 mm × 250 μm). Carrier gas was helium at a flow rate of 0.7 ml/min, injector and MS detector transfer line temperatures were set at 220 °C and 300 °C, respectively. To obtain the total ion chromatograms (GC–MS operating in SCAN mode) of derivatised samples, the following temperature program was followed: oven initially at 100 °C, temperature increased at 5 °C/min to 310 °C, hold 8 min at 310 °C. Under these conditions more than 80 PEE components – in

Fig. 1. Propolis sampling sites.

Table 1
Propolis collection sites and percent yield of propolis ethanolic extraction.

Code	Collection area/year	Geographical location	Ethanolic extract of crude propolis (%w/w)
Greece			
TRI	Trikorfo/2007	Messinia, Southern Peloponnese	27.5
ARF	Arfara/2007	Messinia, Southern Peloponnese	61.2
KAL	Kalavryta/2007	Achaia, North Peloponnese	49.4
MEG	Megalopolis/ 2007	Arcadia, Central Peloponnese	53.2
KAR-I	Karditsa/2006	Thessaly, Central Greece	40.1
KAR-II	Karditsa/2007	Thessaly, Central Greece	58.6
CRE	Aloides/2007	Crete island, Southern Aegean Sea	23.9
TIN	Pirgos/2007	Tinos island, Central Aegean Sea	26.8
SKO	Glossa/2007	Skopelos island, NW Aegean Sea	27.5
LES	Moria/2007	Lesvos island, East Aegean Sea	25.3
Cyprus			
LAR-I	Larnaca/2006	East Cyprus	48.2
LAR II	Larnaca/2007	East Cyprus	40.4

the form of their TMS ethers – were identified using their mass spectra and by reference to NIST 98 (NIST MS search v6.1d) and Wiley 275 (Wiley, New York, NY) mass spectra libraries, as well as by analysing pure standards and by reference to literature. In this way, the qualitative analysis of PEE was achieved, while additionally the flavonoids apigenin, pinocembrin, pinobanksin and pinobanksin-*O*-acetate, the anthraquinones 1,8-dihydroxy-3-methylanthraquinone (chrysophanol), 1,3,8-trihydroxy-6-methylanthraquinone (emodin), 2,7-dihydroxy-5-methoxy-3-methylanthraquinone, and 1,7-dihydroxy-3-methoxy-6-methylanthraquinone, and the terpenic acids isopimaric, abietic and dehydroabietic were quantitatively determined. Abietic acid, apigenine and pinocembrin were identified and quantified by analysing a series of nine standard solutions of the specific compounds. The TMS derivatives of isopimaric and dehydroabietic acids were identified by means of the Wiley 275 mass spectra library (Wiley, New York, NY) and quantified by the abietic acid reference curve. Pinobanksin, and pinobanksin-*O*-acetate were identified by the characteristic ions of their TMS derivatives (Neacsu et al., 2007) and were quantified by means of the pinocembrin reference curve. Among the anhtraquinones detected, chrysophanol and emodin were identified by the characteristic ions of their TMS derivatives (Zuo, Wang, Lin, Guo, & Deng, 2008). Anthraquinones were quantitated by means of the pinocembrin reference curve.

The derivatised samples were rechromatographed by GC–MS operating in selective ion monitoring (SIM) mode. Identification of chromatographic peaks was made by comparing the ±0.05 Rt and ratios of target and qualifier ions of each compound with those of standards, while quantification was carried out by employing 3-(4-hydroxyphenyl)-1-propanol as internal standard. Internal standard quantification was performed based on a series of nine standard mixtures of the individual polyphenols and terpenic acids containing the same amount of internal standard as that of samples. Linearity was obtained for all target compounds detected in samples in the range of quantification limit. In this way, 27 simple polyphenols and two terpenic acids – namely oleanolic and ursolic – were quantitatively determined. Retention times, target and qualifier ions for the trimethylsilyl ethers (TMS) of the 29 compounds and the internal standard are given in Table 2.

2.2.3. Determination of total polyphenols

The total polyphenol content of PEE were determined by the Folin-Ciocalteu colourimetric method, adapted to microscale (Arnous, Makris, & Kefalas, 2002). The results were expressed as mg/g caffeic acid equivalents (CAE).

2.2.4. Measurement of free radical scavenging activity (DPPH· assay)

The ability of PEE constituents to scavenge the "stable" free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) was estimated according to the procedure described by Arnous et al. (2002) and the results were expressed as mmol Trolox® equivalents per g of PEE.

2.2.5. Measurement of reducing power (FRAP assay)

The reducing ability of PEE was determined by the ferric reducing/antioxidant potential (FRAP) assay. This procedure involves the reduction of ferric tripyridyltriazine (FeIII-TPTZ) complex to a blue coloured FeII-TPTZ by sample's antioxidants. For the determination, the protocol described by Arnous et al. (2002) was followed. Ascorbic acid was used as a positive control to construct a reference curve, and the results were expressed as mmol ascorbic acid per g of PEE.

2.2.6. Antimicrobial action assay

The *in vitro* inhibitory activity of propolis extracts against thirteen Gram positive, five Gram negative bacteria and two pathogenic

Table 2
Simple polyphenols and terpenic acids quantitated by GC–MS.

Compound	GC–MS run mode	Target ion (m/z)[a]	Qualifier ions (m/z)[a]
Vanillin	SIM[b]	194	209
Cinnamic acid	SIM	205	220
Tyrosol	SIM	179	267, 282
p-Hydroxybenzoic acid	SIM	267	223, 193
p-Hydroxyphenylacetic acid	SIM	252	296, 281
I.S[c]	SIM	206	191, 179
Phloretic acid	SIM	192	310
Vanillic acid	SIM	297	267, 312
Homovanillic acid	SIM	326	267, 311
o-Coumaric acid	SIM	293	308, 147
Protocatechuic acid	SIM	193	355, 370
3,4-Dihydroxyphenylacetic acid	SIM	384	267, 179
Syringic acid	SIM	327	342, 312
p-Coumaric acid	SIM	308	293, 219
Gallic acid	SIM	281	458, 443
Ferulic acid	SIM	338	323, 308
Caffeic acid	SIM	396	219, 381
Sinapic acid	SIM	368	353, 338
Resveratrol	SIM	444	445, 443
Chrysin	SIM	383	384
Epicatechin	SIM	368	355, 474
Naringenin	SIM	473	296
Catechin	SIM	368	355, 474
Genistein	SIM	473	
Kaempferol	SIM	559	560
Chlorogenic acid	SIM	345	307, 324
Quercetin	SIM	647	559, 575
Myricetin	SIM	735	647, 575
Oleanolic acid	SIM	203	320, 482
Ursolic acid	SIM	203	320, 482
Dehydroabietic acid	SCAN[d]		
Abietic acid	SCAN		
Isopimaric acid	SCAN		
Pinocembrin	SCAN		
Pinobanksin	SCAN		
Pinobanksin-*O*-acetate	SCAN		
Chrysophanol (1,8-dihydroxy-3-methylanthraquinone)	SCAN		
1,6-Dihydroxy-8-methoxy-3-methylanthraquinone	SCAN		
Emodin (1,3,8-trihydroxy-6-methylanthraquinone)	SCAN		
1,7-Dihydroxy-3-methoxy-6-methylanthraquinone	SCAN		
Apigenin	SCAN		

[a] Trimethylsilyl (TMS) ether derivatives of the compounds.
[b] SIM = selective ion monitoring.
[c] I.S. = internal standard, 3-(4-hydroxyphenyl)-1-propanol.
[d] SCAN = total ion current monitoring.

fungi was investigated by the agar well diffusion assay. For this purpose, PEE stock solutions (5% w/v) were serially twofold diluted with 80:20 ethanol:water and the diluted solutions were used for the assay. The sensitivity of the target strains was also tested against nisin, a food grade proteinaceous antibiotic, and their antimicrobial spectra were compared.

For the assay, 15 ml of the appropriate agar medium according to the target strain tested, were added into Petri dishes. The melted and tempered (at 45 °C) agar was previously inoculated with 150 μl of the target cell suspension. The suspensions were prepared by diluting overnight cultures of the target strain into saline solution to, approximately, 10^6 cfu/ml using McFarland turbidity standards (bioMerieux S.A., Marcy l' Étoile, France). The plates were dried for 1 h and then, using a sterile cylinder, wells of 7.0 mm diameter were made and filled up with 100 μl of the diluted PEE solutions. In order to obtain comparable results, all samples were treated under the same conditions in the same plate for each microorganism. The plates were incubated for 24 h at 37 °C and the results were recorded. *Lactobacillus* strains were incubated for 48 h at 37 °C under anaerobic conditions (BBL GasPak system, Becton Dickinson Microbiology Systems, Cockeysville, MD). The inhibitory activity of the samples was detected as a clear zone around the wells. Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the sample that caused a clear (1–3 mm) zone of inhibition. As a negative control, the ethanol: water (80:20 v:v) solution was used. All tests were carried out in triplicate and the results were averaged.

2.2.7. Statistical analysis

All the analyses were duplicated unless otherwise specified, and the results presented are the averages of the obtained values. Data manipulation was performed by means of Microsoft Excel (Microsoft Corp., Redmond, WA). Hierarchical cluster analysis was carried out by Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Herndon, VA)

3. Results and discussion

3.1. PEE chemical composition

3.1.1. Overview

According to the results of the GC–MS analysis, the PEE samples from Greece and Cyprus contained more than 100 compounds, more than 80 of which were identified. Among the identified PEE constituents several compounds with known antioxidant, antiinflammatory and antimicrobial activities, like flavonoids, terpenes, anthraquinones, phenolic acids and their esters were present. The chemical composition of PEE as % of TIC is presented in Table 3 and summarised in Table 4, while the quantitative (w/w) results for individual polyphenols and terpenic acids are given in Table 5.

It is generally accepted that propolis from temperate climatic zones, like Europe, North America and the non-tropical regions of Asia, originate mainly from the bud exudates of *Populus* species and their hybrids, and are rich in flavonoids, phenolic acids and their esters (Bankova, Popova, Bogdanov, & Sabatini, 2002; Bankova et al., 2000), while propolis from tropical regions, where no poplars and birches exist, are rich in prenylated benzophenons, diterpenes and flavonoids (Ahn et al., 2007; Bankova, 2005; Banskota et al., 2001). From the analytical results obtained, it is obvious that, besides differences among individual constituents, the Greek and Cypriot propolis samples share characteristics that differentiate them from typical European propolis, like the presence of anthraquinones and terpenes in significant amounts, both when expressed as % of TIC and as mg/g of PEE (Tables 4 and 5), and the relatively low abundance of phenolic acids and their esters. By performing hierarchical cluster analysis in the data of Table 4, after excluding aliphatic acids and alcohols, the propolis samples were divided into two groups: one comprised of samples from central Peloponnese (MEG) and central Greece (KAR-I and KAR-II), which contained low amounts of terpenes and were rich in anthraquinones, flavonoids, phenolic acids and their esters, and a second one comprised of the rest of PEE samples (graph not shown).

3.1.2. Terpenes

The majority of the 12 PEE samples studied contained significant amounts of terpenes, ranging from 1.43% to 41.87% of TIC, while in seven samples, they comprised more than 30% of TIC (Tables 3 and 4). Terpenoids have been also observed in propolis from Mediterranean areas like Sicily, Turkey and Algeria (Bankova et al., 2002; Velikova et al., 2000), and Greece (Melliou & Chinou, 2004; Melliou et al., 2007). Based on pollen analysis, Melliou and Chinou (2004) proved that a significant source of propolis from mainland Greece was *Coniferae* trees – especially *Pinus* sp. – the resin of which is rich in terpenic acids like abietic, dehydroabietic and

Table 3
Composition of propolis extracts from Greece and Cyprus assessed by GC–MS as trimethylsilyl ethers derivatives (% of total ion current[a]).

Compound[b]	Rt[c]	Composition											
		TRI	ARF	KAL	MEG	KAR-I	KAR-II	CRE	TIN	SKO	LES	LAR-I	LAR-II
Alcohols													
Ethylene glycol	5.77	0.21	0.41	0.32	0.45	0.39	0.37	0.52	0.24	0.29	0.24	0.43	0.62
Phenethyl alcohol	6.07	–	–	0.30	0.69	–	–	–	–	–	–	–	–
Glycerol	6.99	0.61	1.43	0.86	0.26	1.41	0.45	6.80	3.21	1.42	1.24	1.62	1.12
Aliphatic acids													
Succinic acid	7.69	0.13	–	–	–	–	–	0.27	0.16	–	0.08	–	0.21
Malic acid (hydroxybutanedioic acid)	11.67	0.10	0.05	0.09	–	0.34	–	0.48	0.32	0.26	0.11	–	–
Azelaic acid	18.24	–	–	–	–	–	–	0.40	–	–	–	–	–
Hexadecanoic acid	22.96	0.28	0.27	0.39	–	–	0.28	0.88	0.51	0.34	0.78	0.63	1.02
Oleic acid	26.04	0.65	0.36	0.57	0.43	0.97	0.51	1.00	0.72	1.37	1.10	0.80	1.65
2-Hexenedioic acid	32.96	0.52	0.33	–	–	–	–	0.41	0.47	0.70	0.98	–	–
Aromatic acids													
Benzoic acid	6.42	–	–	–	–	0.57	1.99	–	–	–	–	–	–
3,4-Methylenedioxycinnamic acid	6.73	0.31	0.57	0.49	0.68	0.53	0.58	0.80	0.41	0.47	0.11	0.64	0.93
Cinnamic acid	12.70	–	–	0.24	0.76	–	–	–	–	–	–	–	–
3,4-Dimethoxycinnamic acid	22.71	–	0.29	0.28	0.74	1.13	0.63	–	–	–	0.21	–	–
Ferulic acid	23.68	–	0.42	–	0.63	0.82	0.32	–	–	–	0.09	–	–
Caffeic acid	24.84	0.25	0.85	–	–	1.56	0.81	–	–	0.20	0.47	–	–
p-Coumaric acid	28.57	0.54	0.82	2.85	0.48	2.13	0.80	–	–	–	0.83	–	–
Δ-9-Tetrahydrocannabinol acid	30.53	0.20	0.43	0.54	1.75	0.66	0.40	–	0.29	–	0.36	–	–
Gallic acid	33.93	–	–	0.81	1.73	0.63	–	–	–	–	–	–	–
Phloroglucinic acid	35.47	0.20	0.24	1.44	2.15	1.11	0.53	–	0.74	0.41	–	0.94	1.83
Esters													
Ethyl benzoate	5.18	–	–	0.38	–	–	–	–	–	–	–	–	–
3-Methyl-2-butenyl isoferulate	28.68	0.19	0.34	–	–	1.22	0.54	–	–	–	–	–	–
Cinnamyl cinnamate	29.45	0.27	1.49	–	–	1.17	0.55	–	0.06	0.20	1.16	–	–
Cinnamic acid ester	31.15	–	–	–	–	–	–	–	–	–	–	5.21	7.46
Benzyl ferulate	33.48	0.45	0.28	0.20	0.48	1.16	1.79	–	–	0.21	–	–	–
Phenylethyl caffeate (CAPE)	35.24	1.18	2.31	1.26	2.05	3.17	1.56	–	0.70	1.30	0.77	1.26	1.49
Cinnamyl caffeate	38.41	0.24	0.47	0.89	1.17	1.84	1.46	–	–	0.42	1.36	1.31	–
Flavonoids													
Pinostrobin (chalcone)	31.25	0.23	1.37	0.43	0.84	0.35	0.72	–	–	–	0.33	–	–
Pinocembrin	31.50	5.18	5.83	6.83	11.52	8.96	18.03	4.01	5.24	4.44	0.92	0.66	3.41
Pinobanksin	32.33	0.40	1.08	6.21	3.70	3.38	2.59	–	–	1.52	1.39	1.17	0.53
Pinobanksin-3-*O*-acetate	33.57	2.16	5.35	1.96	5.84	7.61	8.97	0.79	0.48	2.94	4.86	2.77	1.92
Chrysine	34.06	0.79	1.13	0.46	0.92	3.06	4.50	–	0.51	1.73	0.73	–	1.35
Galangin	34.57	0.36	0.90	1.09	1.92	1.25	0.39	–	0.63	0.48	0.23	–	0.15
Pinobanksin isobutanoate	34.98	0.64	1.04	–	1.30	1.23	0.73	–	0.40	0.59	0.24	1.02	0.5
Naringenin	36.26	0.58	1.41	1.18	2.64	2.47	0.84	–	–	1.20	0.35	0.41	1.05
Kaempferol	39.19	–	–	0.85	0.88	–	–	–	–	–	1.63	–	–
Apigenine	39.90	0.2	0.28	0.22	1.61	0.28	0.23	–	–	–	–	–	–
Quercetin	39.94	0.21	0.25	0.15	–	0.30	0.18	–	–	–	–	–	–
Anthraquinones													
Chrysophanol (1,8-dihydroxy-3-methylanthraquinone)	34.48	1.88	5.08	6.87	12.55	9.93	4.83	–	0.31	2.36	0.17	0.10	0.79
1,6-Dihydroxy-8-methoxy-3-methylanthraquinone)	34.69	–	–	–	–	–	–	–	–	–	1.08	–	–
Emodin (1,3,8-trihydroxy-6-methylanthraquinone)	35.55	1.57	3.33	6.65	10.30	4.37	6.04	–	–	1.49	1.18	–	0.29
1,7-Dihydroxy-3-methoxy-6-methylanthraquinone	35.67	–	–	0.64	1.44	–	0.51	0.54	–	0.65	0.32	–	–
Ketones													
5-Hydroxy-1,7-diphenyl-3-heptanone	29.58	–	–	–	–	–	–	–	–	–	–	–	1.78
(3e,5e,7e)-6-Methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-one	30.74	3.79	2.52	–	0.30	–	0.40	1.62	2.40	2.73	–	–	0.90
Sugars													
D-Fructose	18.85	5.78	2.98	0.74	2.17	6.43	6.77	16.99	11.84	6.26	15.65	6.31	11.5
Sorbose	19.10	0.31	–	–	–	0.38	–	1.22	0.69	0.31	0.79	0.98	0.87
Inositol	19.39	–	–	–	–	–	–	–	–	0.34	–	–	1.15
D-Xylose	20.57	–	–	–	–	0.46	–	–	–	–	4.71	–	–
D-Glucose	20.61	1.30	1.20	–	–	1.85	2.60	3.37	1.74	0.90	–	2.14	2.21
D-Glucitol	21.55	–	–	–	–	–	–	0.75	0.47	–	0.28	4.42	–
D-Mannose	22.41	1.90	2.02	–	–	3.26	3.96	4.82	2.86	1.08	8.27	2.85	4.05
Sucrose	33.69	0.76	0.52	–	–	1.51	3.46	1.36	1.41	0.55	19.65	1.24	–
Terpenes													
Thymol	7.80	–	0.11	0.10	0.36	–	–	–	–	–	–	–	–
Menthol	24.67	0.46	0.25	–	–	–	–	–	0.29	0.42	–	–	–
Thunbergol	27.04	–	–	–	–	–	–	–	0.34	–	–	–	–
Pimaric acid	28.03	2.45	1.42	0.45	–	0.47	0.60	2.23	3.10	1.33	–	0.11	–
Totarol	28.19	4.33	4.49	–	–	0.31	0.47	0.99	1.65	2.20	–	1.52	–
Phytol	28.23	3.90	2.05	–	–	0.61	0.60	2.71	2.57	3.61	–	2.91	0.31
Dehydroabietic acid	29.03	0.49	0.21	2.85	0.01	0.07	0.04	0.20	0.17	0.27	1.57	1.74	3.05
Abietic acid	29.50	0.12	0.03	32.59	0.06	0.03	–	0.42	0.52	0.17	0.79	1.00	1.73
Isopimaric acid	32.23	27.99	19.02	5.88	1.00	1.78	2.93	14.96	22.87	28.96	1.50	0.88	1.81

(*continued on next page*)

Table 3 (*continued*)

Compound[b]	Rt[c]	Composition											
		TRI	ARF	KAL	MEG	KAR-I	KAR-II	CRE	TIN	SKO	LES	LAR-I	LAR-II
Coeluting terpenes (*trans*-caryophyllene, geranyl acetone, caryophyllene based diterpene)	36.90	–	–	–	–	–	–	–	–	–	–	24.90	–
Aristolone	42.56	0.20	1.79	–	–	–	–	2.03	1.16	0.44	0.28	0.54	0.74
α-Amyrin	43.00	–	–	–	–	–	–	3.58	2.04	–	–	–	–
Hop-22(29)-en-3-beta-ol	43.11	–	–	–	–	–	–	5.63	2.98	–	0.21	–	–
Urs-12-en-24-oic acid, 3-oxo-, methyl ester	44.30	–	–	–	–	–	–	1.42	0.76	–	–	1.35	0.61
Others													
D-Glucosamine	17.06	–	–	0.26	0.53	–	–	–	–	–	–	0.39	–
2-Methyl quinoline (quinaldine)	29.11	1.35	0.98	0.94	0.34	–	–	0.95	1.32	1.52	0.64	–	–
p-Phenylazodiphenylamine	31.01	0.33	0.79	4.77	5.32	2.48	1.78	–	–	0.43	–	–	–
7,8-Dimethylbenzo[b]naphtho[2,3-d]thiophene	31.69	–	–	0.81	1.04	–	–	0.63	0.67	–	–	–	1.21
5,10-Dihydro-1,2,3,4-tetraphenylbenzocyclooctene	32.10	–	–	–	–	–	0.32	–	0.44	–	–	2.05	3.03
Indole-2-acetic acid	32.82	–	–	–	–	–	0.48	0.74	1.05	–	0.31	0.44	0.77
Phenanthrene	33.31	1.21	2.68	0.58	2.81	4.04	3.37	–	–	0.73	0.74	–	–
Scopolin	33.82	–	–	–	–	0.32	1.03	–	–	–	–	–	–
Thianthrene	34.26	0.31	0.34	–	0.75	–	–	–	0.24	–	–	–	–
2-(3′-Hydroxyphenylamino)-5-methyl-4-oxo-3,4-dihydropyrimidine	35.33	0.55	–	–	–	–	–	–	0.35	–	0.21	0.94	–
1,2,3-Triphenyl azulene	37.10	–	–	–	–	–	–	–	–	–	0.50	0.57	–
p-Tert-butyl-phenol	37.65	–	–	–	0.68	–	0.72	–	–	–	–	2.81	–

–: not detected.
[a] The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.
[b] Compound names do not include the trimethylsilyl (TMS) substituents.
[c] Retention times in min on a HP5 MS column.

Table 4
Main classes of the PEE constituents (% of TIC[a]).

Code	Collection site	Alcohols	Aliphatic acids	Phenolic acids	Phenolic acid esters	Anthraquinones	Flavonoids	Sugars	Terpenes
	Greece								
TRI	Trikorfo	0.82	1.68	1.50	2.33	3.45	10.75	10.05	39.94
ARF	Arfara	1.84	1.01	3.62	4.89	8.41	18.64	6.72	30.07
KAL	Kalavryta	1.48	1.05	6.65	2.73	14.16	19.38	0.74	41.87
MEG	Megalopolis	1.40	0.43	8.92	3.70	24.29	31.17	2.17	1.43
KAR-I	Karditsa	1.80	1.31	8.57	8.56	14.30	28.89	13.89	3.27
KAR-II	Karditsa	0.82	0.79	4.07	5.90	11.38	37.18	16.79	4.64
CRE	Rethymno	7.32	3.44	0.80	0.00	0.54	4.80	28.51	34.17
TIN	Tinos	3.45	2.18	1.44	0.76	0.31	7.26	19.01	38.45
SKO	Skopelos	1.71	2.67	1.08	2.13	4.50	12.90	9.44	37.40
LES	Lesvos	1.48	3.05	2.07	3.29	2.75	10.68	49.35	4.35
	Cyprus								
LAR-I	Larnaca	2.05	1.43	1.58	7.78	0.10	6.03	17.94	34.95
LAR-II	Larnaca	1.74	2.88	2.76	8.95	1.08	8.91	20.23	8.25

[a] TIC = total ion current. The ion current generated depends on the characteristics of the compound concerned and cannot be considered as a true quantitation.

isopimaric (Joye & Lawrence, 1967). With the exception of samples from Central Peloponnese (MEG) and Central Greece (KAR-I, KAR-II), these terpenic acids were among the major terpenes determined in PEE from Greece and Cyprus (Tables 3 and 5). Significant amounts of resin terpenic acids have also been reported in propolis from Turkish Anatolia (Kartal, Kaya, & Kurucu, 2002).

An interesting finding was the presence of the diterpene totarol, which was identified by means of the m/z ions of its TMS derivative (Cox, Yamamoto, Otto, & Simoneit, 2007). Totarol, which was detected in seven Greek and one Cypriot propolis, comprising 0.31–4.3% of TIC (Table 3), is present in southern hemisphere conifers, characterizing – together with other diterpenes – the tropical propolis (Cox et al., 2007). Totarol was also detected in Greek propolis by Melliou and Chinou (2004) and Melliou et al. (2007) – the first record of totarol in European propolis. Totarol is a known antimicrobial agent against Gram positive bacteria (Cowan, 1999), and totarol isolated from Greek propolis showed a specific activity against *S. aureus* and *S. epidermidis*, comparable to that of standard antibiotics (Melliou & Chinou, 2004).

The sesquiterpene *trans*-caryophyllene together with a caryophyllene based diterpene and geranyl acetone constituted the major fraction – 24.9% of TIC – of one Cyprus propolis, namely LAR-I (Table 3). High levels of *trans*-caryophyllene and caryophyllene oxide at levels up to 2.49% and 7.21% of TIC, respectively, were reported in terpenes-rich propolis from Turkish Mediterranean coast (Sahinler & Kaftanoglu, 2005).

Phytol, at levels from 0.3% to 3.9%, was also observed in the majority of samples (Table 3). Phytol is an acyclic diterpene alcohol, present in all plants as chlorophyll esters. It is a precursor for vitamins E and K_1, and has been proved to possess antimicrobial properties (Inoue et al., 2005).

Aristolone was detected in propolis from Greek islands (CRE, SKO, TIN, LES), Cyprus (LAR-I, LAR-II) and South Peloponnese (TRI, ARF) (Table 3). Propolis from Tinos (TIN) and Crete (CRE) islands contained additionally α-amyrin, one hopenol and one ursenoic acid isomers (Table 3). In Tinos (TIN), thunbergol was detected in propolis, which was reported for the first time in Turkish propolis from Kazan (Kartal et al., 2002).

As far as terpenes are concerned, propolis balsams from Greece and Cyprus exhibit similarities with propolis from Eastern Mediterranean, and differences from the typical European ones.

Table 5
Simple polyphenols[a] and terpenic acids quantitated by GC–MS in propolis from Greece and Cyprus (mg/g dry ethanolic extract). Each value represents the average of two determinations. Standard deviations were less than 10%.

Compounds	TRI	ARF	KAL	MEG	KAR-I	KAR-II	CRE	TIN	SKO	LES	LAR-I	LAR-II
Polyphenols												
Vanillin	0.03	0.04	0.01	0.04	0.21	0.97	tr[b]	nd[c]	0.05	0.11	nd	nd
Cinnamic acid	0.31	0.22	1.68	4.00	0.79	1.67	0.05	0.04	0.15	0.05	0.04	0.10
p-OH benzoic acid	0.08	0.05	0.06	0.06	0.07	0.08	0.10	0.11	0.11	0.19	0.05	0.07
p-OH phenylacetic acid	0.04	nd	0.02	0.02	0.02	nd	0.06	0.06	0.03	0.02	0.03	0.03
Phloretic acid	0.08	0.09	0.03	0.05	0.08	0.08	0.19	0.28	0.07	0.14	0.04	0.17
Vanillic acid	0.05	0.05	0.06	0.07	0.05	0.08	0.05	0.05	0.06	0.05	0.04	0.04
o-Coumaric acid	0.06	0.03	0.04	0.02	nd	nd	0.05	0.07	0.05	nd	tr	0.05
Protocatechuic acid	0.10	0.07	0.05	0.06	0.06	0.08	0.13	0.07	0.33	0.11	0.10	0.17
Syringic acid	0.03	nd	0.04	0.05	0.03	0.03	0.04	0.04	0.04	0.05	0.03	0.04
p-Coumaric acid	0.44	0.75	0.25	0.31	0.75	2.18	0.07	0.08	0.67	0.40	0.12	0.19
Gallic acid	0.06	0.06	0.04	nd	0.05	0.04	0.04	0.04	0.07	0.10	0.04	0.11
Ferulic acid	0.46	0.71	0.79	1.81	1.07	0.84	0.09	0.11	0.26	0.16	0.10	0.13
Caffeic acid	1.74	3.87	0.22	0.21	3.55	3.29	0.14	0.15	1.14	6.70	0.16	0.31
Chrysin	45.44	87.86	118.5	145.7	96.75	40.38	0.26	1.27	34.77	19.64	0.24	5.94
Epicatechin	0.06	0.10	0.04	0.06	0.04	0.04	0.05	0.05	0.06	0.07	0.05	0.04
Naringenin	0.31	0.59	0.64	0.94	0.44	0.38	0.22	0.13	0.58	0.42	0.07	0.28
Catechin	0.08	0.10	0.07	0.08	0.07	0.07	0.07	0.07	0.08	0.07	0.06	0.07
Genistein	0.05	0.06	nd	0.06	0.05	nd	nd	nd	nd	0.06	0.04	0.07
Kaempferol	0.92	1.74	3.75	3.42	1.73	1.80	0.08	0.21	0.80	2.35	0.08	1.28
Chlorogenic acid	nd	0.08	0.05	0.05	0.02	nd	0.12	0.14	0.11	0.13	0.09	0.10
Quercetin	0.20	0.36	nd	0.41	nd	nd	0.05	0.06	0.13	0.35	0.05	0.09
Apigenine	9.20	15.85	11.89	13.03	12.49	6.63	nd	nd	0.25	2.96	nd	nd
Pinocembrin	48.03	33.34	36.90	51.60	43.71	104.8	16.47	38.07	32.45	16.47	3.70	20.26
Pinobanksin	3.68	6.21	33.74	16.52	16.59	16.81	0.32	0.51	11.31	24.24	0.55	1.59
Pinobanksin-3-*O*-acetate	19.98	30.76	11.09	26.81	37.09	52.18	3.39	0.38	21.82	55.72	4.29	5.67
Chrysophanol (1,8-dihydroxy-3-methylanthraquinone)	16.85	28.12	37.46	53.96	46.89	36.37	nd	nd	16.19	2.87	0.10	0.74
1,6-Dihydroxy-8-methoxy-3-methylanthraquinone	3.16	4.99	5.98	8.20	5.83	2.22	nd	nd	3.22	0.33	nd	nd
Emodin (1,3,8-trihydroxy-6-methylanthraquinone)	10.49	13.77	32.93	36.61	21.04	29.95	0.30	4.70	7.87	20.83	1.80	4.08
1,7-Dihydroxy-3-methoxy-6-methylanthraquinone	1.91	1.40	4.79	9.30	4.03	3.27	nd	0.78	1.72	0.16	tr	1.12
Terpenic acids												
Dehydroabietic acid	20.59	5.68	27.03	0.06	1.53	1.13	3.55	5.60	9.56	82.34	17.75	46.97
Abietic acid	4.74	1.05	345.1	1.42	0.96	0.28	7.64	16.70	5.75	49.42	16.57	23.44
Isopimaric acid	259.6	108.8	1.94	4.45	8.66	17.05	61.76	166.3	210.1	18.46	5.50	17.44
Oleanolic acid	1.26	0.91	0.36	0.44	0.53	2.78	0.35	0.37	1.96	11.54	0.46	1.67
Ursolic acid	0.35	0.41	0.44	0.86	0.45	0.49	0.26	0.32	0.54	0.29	0.28	tr
Sum of polyphenols	163.8	231.3	301.2	373.5	293.5	304.3	22.34	47.47	134.4	154.8	11.87	40.63
Sum of terpenic acids	286.5	116.9	374.9	7.23	12.13	21.73	73.56	189.3	227.9	162.1	40.56	89.52

[a] Among the single polyphenols determined, traces of tyrosol, homovannilic acid, 3,4-dihydroxy-phenylacetic acid, sinapic acid, resveratrol and myricetin observed in some samples were not included in the Table.
[b] tr = trace (<0.01 mg/g).
[c] nd = not detected.

3.1.3. Anthraquinones

A characteristic of the propolis samples studied was the presence of anthraquinones at levels ranging from 0.54% to 26.34% of TIC, and 0.3–108.1 mg/g of PEE (Tables 3 and 5). Among the anthraquinones detected, the known bioactive compounds chrysophanol and emodin (Tang, Wan, Zhu, Chen, & Huang, 2008) predominated. Anthraquinones have also been detected in propolis from Egypt (Abd El Hady & Hegazi, 2002) and Turkey, where chrysophanol at levels as high as 4.54% and 15.15–20.82% of TIC were reported (Silici & Kutluca, 2005; Silici, Ünlü, & Vardar-Ünlü, 2007).

3.1.4. Flavonoids

Propolis from Greece and Cyprus contained flavonoids at levels of 4.8–37.18% of TIC, and 8.8–182.6 mg/g of PEE (Tables 4 and 5), with the higher values observed in propolis from central Peloponnese (MEG) and central Greece (KAR-I, KAR-II) and the lower in PEE from the islands of Crete (CRE) and Tinos (TIN) (Table 4). Flavonoids are synthesised by plants as a response to environmental stress and microbial infections, and are known to have antioxidant, antiinflammatory and antimicrobial properties (Bankova, 2005; Cowan, 1999; Cushnie & Lamb, 2005; Pietta, 2000). Among the compounds characterising the European propolis, the flavonoids pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin, and galangin were present in the majority of the propolis studied (Tables 3 and 5).

3.1.5. Phenolic acids and their esters

Phenolic acids and esters comprised 3.21–17.13% of TIC, the higher values being observed in propolis from Cyprus (LAR-I, LAR-II), central Peloponnese (MEG) and Central Greece (KAR-I, KAR-II) and the lower in propolis from Crete (CRE) island (Table 4). The phenolic acids observed on a w/w basis ranged from 0.75 to 9.34 mg/g of PEE (Table 5). As these compounds possess significant antibacterial, antiinflammatory, hepatoprotective and antioxidant activity (Bankova, 2005), their presence in the PEE studied is considered beneficial. Especially the presence of caffeic acid phenylethyl ester (CAPE), which has been reported to show antitumour activity (Bankova, 2005), at levels 0.70–3.17% of TIC (Table 3).

3.2. Total polyphenols

The total polyphenol content of the propolis studied ranged between 80.2 and 338.5 mg GAE/g PEE (Table 6). These values are within the range of 31.2–299 mg GAE/g PEE, reported for propolis from several regions of the world (Kumazawa, Hamasaka, & Nakayama, 2004). The higher polyphenol content was observed in PEE from Central and North Peloponnese (MEG, KAL) and Central Greece (KAR-I, KAR-II). Total polyphenol content correlated very well ($p < 0.01$) with DPPH$^{\cdot}$ antioxidant activity ($R = 0.905$) and reducing power ($R = 0.832$). In relation to main classes of PEE con-

Table 6
Total polyphenol content, free radical scavenging capacity on DPPH· and reducing power in propolis extracts from Greece and Cyprus. Values are mean ± SD obtained from analyses in triplicate.

Code	Collection site	Total poyphenols[a] (mg CAE[b]/g PEE[c])	DPPH· scavenging capacity[d] (mmol Trolox/g PEE)	Reducing power[e] (mmol AAE[f]/g PEE)
TRI	Trikorfo	146.2 ± 7.3	0.60 ± 0.04	3.13 ± 0.16
ARF	Arfara	184.6 ± 7.4	0.55 ± 0.03	3.09 ± 0.22
KAL	Kalavryta	250.6 ± 17.5	0.76 ± 0.05	3.13 ± 0.25
MEG	Megalopolis	338.5 ± 13.2	1.11 ± 0.07	3.35 ± 0.27
KAR-I	Karditsa	283.5 ± 21.3	1.05 ± 0.04	3.34 ± 0.20
KAR-II	Karditsa	322.0 ± 13.1	0.99 ± 0.03	3.24 ± 0.13
CRE	Rethymno	80.2 ± 3.2	0.33 ± 0.03	2.14 ± 0.11
TIN	Tinos	107.7 ± 5.4	0.65 ± 0.03	2.75 ± 0.08
SKO	Skopelos	146.2 ± 10.2	0.62 ± 0.02	2.89 ± 0.14
LES	Lesvos	136.3 ± 8.2	0.45 ± 0.02	2.65 ± 0.19
LAR-I	Larnaca Cyprus	85.7 ± 5.1	0.46 ± 0.03	2.41 ± 0.10
LAR-II	Larnaca Cyprus	100.4 ± 7.2	0.58 ± 0.03	2.63 ± 0.08

[a] Total polyphenol content was determined by the Folin-Ciocalteu assay.
[b] CAE = caffeic acid equivalent.
[c] PEE = propolis ethanolic extract.
[d] Free radical scavenging capacity was measured with DPPH· (1,2-diphenyl-2-picrylhydrazyl) radical.
[e] Reducing power was determined by the ferric reducing antioxidant power (FRAP) assay.
[f] AAE = ascorbic acid equivalent.

stituents (Table 4), total polyphenol content correlated very well ($p < 0.01$) with phenolic acids ($R = 0.848$), anthraquinones ($R = 0.923$) and flavonoids ($R = 0.957$), while in relation to individual polyphenols and terpenic acids (Table 5), total polyphenols correlated very well ($p < 0.01$) with cinnamic acid ($R = 0.836$), vanillic acid ($R = 0.791$), ferulic acid ($R = 0.918$), chrysin ($R = 0.818$), pinocembrin ($R = 0.750$), kaempferol ($R = 0.741$), apigenine ($R = 0.735$), all the anthraquinones ($R = 0.807–0.956$), and ursolic acid ($R = 0.760$).

3.3. Antioxidant activity (DPPH·) assay

The propolis extracts studied exhibited significant activity towards scavenging DPPH· radicals, ranging from 0.33 to 1.11 mmol Trolox equivalents/g PEE (Table 6). There was a trend for higher DPPH· values in propolis from central Peloponnese (MEG) and central Greece (KAR-I, KAR-II).

DPPH· assay values correlated very well ($p < 0.01$) with total polyphenols as mentioned in Section 3.2, and additionally correlated very well ($p < 0.01$) with reducing power assay values ($R = 0.838$). In relation to the main classes of PEE constituents (Table 4), DPPH· values correlated very well with phenolic acids ($R = 0.838$), anthraquinones ($R = 0.859$) and flavonoids ($R = 0.905$). Among the polyphenols determined quantitatively (Table 5) DPPH· values correlated very well ($p < 0.01$) with cinnamic acid ($R = 0.775$), ferulic acid ($R = 0.857$), pinocembrin ($R = 0.714$) and the anthraquinones ($R = 0.724–0.876$), while also correlating well ($p < 0.05$) with vanillic acid, chrysin and the terpene ursolic acid.

3.4. Reducing power (FRAP) assay

The PEE reducing power values, expressed as mmol ascorbic acid equivalents (AAE), ranged between 2.14 and 3.35 mmol AAE/g PEE (Table 6), with relatively higher values observed in mainland Greece samples (Peloponnese and Central Greece) and lower in Greek islands and Cyprus. The reducing power values correlated very well ($p < 0.01$) with total polyphenols and DPPH assay values as mentioned in Sections 3.2 and 3.3, respectively. In relation to the main classes of PEE constituents (Table 4), the reducing power values correlated very well ($p < 0.01$) with phenolic acids ($R = 0.711$), anthraquinones ($R = 0.799$) and flavonoids ($R = 0.823$), while in relation to individual polyphenols and terpenic acids (Table 5) the reducing power values correlated very well ($p < 0.01$) with ferulic acid ($R = 0.811$), chrysin ($R = 0.802$), apigenin ($R = 0.804$), and the anthraquinones ($R = 0.750–0.886$), while correlating well ($p < 0.05$) with cinnamic acid, vanillic acid, kaempferol, pinocembrin, and ursolic acid.

3.5. Antimicrobial activity

The antimicrobial activity of Greek and Cypriot propolis ethanolic extracts were tested against eighteen bacterial strains, both pathogenic and non-pathogenic, as well as against two pathogenic fungi and the results are presented in Table 7. The inhibitory spectra of PEE were compared with the inhibitory spectrum of nisin, a known food grade antimicrobial peptide (bacteriocin) produced by *Lactococcus lactis* subsp. *lactis* and used as natural preservative in processed cheese, milk, canned foods, pasteurised liquid egg, flour products and elsewhere.

The sensitivity of Gram positive bacteria to PEE varied among the strains tested and the PEE used. *S. aureus*, *S. epidermidis, B. cereus* and *L. monocytogenes* strains were sensitive against all the PEE tested. Among the pathogenic strains the lowest Minimum Inhibitory Concentration (MIC) of PEE was observed for the two *L. monocytogenes* strains and both *B. cereus* strains while the highest MIC was observed for the (non-pathogenic) *S. epidermidis* (Table 7). Although all the PEE samples inhibited all the Gram positive pathogenic bacteria tested, their inhibitory activity against six strains of the lactic acid bacteria group was rather occasional (Table 7). The PEE samples did not inhibit *L. delbrueckii* subsp. *delbrueckii* and *L. plantarum*, but they did inhibit *L. fermentum* and *L. helveticus* while *L. bulgaricus* and *L. casei* were inhibited only by the KAL and LAR-I (Table 7) samples which exhibited the strongest antibacterial activity. Bozcuk-Erdem and Ölmez (2004) reported no inhibition of *L. casei* RSKK 591 with four Turkish propolis extracts, while three Turkish and one Brazilian propolis extracts inhibited *L. acidophilus* ATCC 4356 (Koru et al., 2007). However, it should be pointed out that the MIC of all the PEE tested was lower for pathogenic bacteria like *S. aureus, L. monocytogenes* and *B. cereus* than for lactic acid bacteria (Table 7). This observation suggests that low concentrations of propolis extracts could be possibly used in fermented products, aiming to selectively inhibit the growth of pathogenic bacteria allowing the survival of starter culture strains like lactic acid bacteria.

Regarding the sensitivity of the Gram negative bacteria tested only the KAL sample from North Peloponnese and LAR-I sample from Cyprus inhibited three out of four Gram negative bacteria that were tested. KAL PEE was very rich in diterpenic acids, like

Table 7
Minimum inhibitory concentrations (MIC)[a] of PEE and nisin towards selected strains of Gram (+), Gram (−) bacteria and yeasts.

Target strains	Propolis extracts (5% w/v)												NISIN
	TRI	ARF	KAL	MEG	KAR-I	KAR-II	CRE	TIN	SKO	LES	LAR-I	LAR-II	
	MIC (mg/mL)												MIC (IU/mL)
Shigella dysenteriae	–	–	2.50	–	–	–	–	–	–	–	2.50	–	–
Salmonella typhimurium	–	–	2.50	–	–	–	–	–	–	–	2.50	–	–
E. coli O157:H7	–	–	5.00	–	–	–	–	–	–	–	5.00	–	–
Y. enterocolitica	–	–	–	–	–	–	–	–	–	–	–	–	–
E. aerogenes	–	–	2.50	–	–	–	–	–	–	–	2.50	–	–
S. aureus I	0.30	0.30	0.15	0.60	0.30	0.30	0.60	0.60	0.30	0.30	0.15	0.60	–
S. aureus II	0.30	0.30	0.15	0.60	0.30	0.30	0.60	0.60	0.30	0.30	0.15	0.60	2000
S. epidermidis	1.25	1.25	0.30	1.25	0.60	1.25	2.50	2.50	1.25	1.25	0.30	1.25	–
B. cereus I	0.08	0.04	0.02	0.08	0.08	0.08	0.08	0.08	0.15	0.08	0.02	0.02	–
B. cereus II	0.08	0.04	0.04	0.08	0.08	0.08	0.08	0.08	0.15	0.08	0.02	0.02	–
L. monocytogenes I	0.08	0.08	0.08	0.15	0.08	0.08	0.08	0.15	0.15	0.08	0.04	0.30	–
L. monocytogenes II	0.04	0.08	0.08	0.15	0.08	0.08	0.08	0.15	0.15	0.04	0.04	0.30	2000
L. delbrueckii subsp. *delbrueckii*	–	–	–	–	–	–	–	–	–	–	–	–	1000
L. bulgaricus	–	–	0.60	–	–	–	–	–	–	–	0.60		250
L. fermentum	2.50	2.50	0.60	2.50	2.50	2.50	2.50	2.50	2.50	2.50	0.60	2.50	250
L. casei	–	–	0.30	–	–	–	–	–	–	–	0.60	–	125
L. plantarum	–	–	–	–	–	–	–	–	–	–	–	–	500
L. helveticus	0.60	0.60	0.15	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.15	0.60	125
Candida albicans	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	–
Candida tropocalis	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	–

[a] Values are the average of three measurements.

dehydroabietic, abietic and isopimaric (Tables 3 and 5) known to contribute to the antibacterial activity of propolis (Bankova et al., 1996; Popova, Silici, Kaftanoglu, & Bankova, 2005), accompanied with significant amounts of flavonoids, anthraquinones, phenolic acids and esters (Table 4). LAR-I PEE was the only sample that contained high amounts of caryophyllene and its derivative, as well as geranyl acetone and increased concentrations of phenolic acids and their esters (Tables 3 and 4). Caryophyllene has known antiinflammatory and antifungal activities (Sabulal et al., 2006). Therefore it seems that the presence of significant amounts of terpenoids – combined with other bioactive compounds – is responsible for the broad spectrum of microorganisms inhibited by the KAL and LAR-I propolis samples. Melliou et al. (2007) reported that the volatiles of Greek propolis inhibited four different species of Gram negative bacteria (*E. coli, E. cloacae, K. pneumoniae, P. aeruginosa*). Ethanolic extract of Bulgarian propolis inhibited 90.9% of the Gram negative bacteria tested (Boyanova, Kolarov, Gergova, & Mitova, 2006) while Bankova et al. (1996) found no inhibitory activity of Brazilian and Bulgarian propolis extracts against a strain of the Gram negative bacterium *E. coli*. Also, Brazilian and Korean propolis extracts inhibited the Gram negative bacterium *S. typhimurium* ATCC 13311, but failed to inhibit the Gram negative *Pseudomonas aeruginosa* ATCC 15523 (Choi et al., 2006).

In general, with the exception of the terpene-rich KAL and LAR-I samples which presented the strongest microbicidal activity, no correlation could be established between PEE composition and their antimicrobial spectrum since similar antimicrobial activities were observed among samples with entirely different chemical composition. Although more than 300 constituents have been identified in propolis samples, biological activity is mainly due to a few classes of substances such as flavonoids, terpenes, phenolic acids and their esters, which have been reported to possess antimicrobial activities, and in combination considered to act synergistically (Bankova, 2005; Marcucci, 1995). This could offer an explanation for the selective and strong antimicrobial activity of propolis from different regions of Greece and Cyprus, as their ethanolic extracts were very rich in terpenes and aromatic compounds (flavonoids, anthraquinones, phenolic acids and esters) the combined levels of which comprised 23.1–84.8% of propolis extracts (Table 4). It also confirms the known ability of bees to collect the best agents to protect their hives against bacterial and fungal infections.

Concerning the antimicrobial activity of nisin, it did not inhibit any of the Gram negative bacteria and the fungi tested. Moreover, some strains of the Gram positive pathogenic bacteria, like *L. monocytogenes*, *B. cereus* and *S. aureus,* were not inhibited by nisin but they were by all PEE samples applied. On the contrary, all the *Lactobacillus* strains were sensitive to nisin (Table 7), a known disadvantage for applying nisin in fermented foods as, along with spoilage and pathogenic bacteria, desirable starter cultures (e.g., lactic acid bacteria) are also inhibited.

Regarding the *in vitro* antimicrobial spectra of nisin and propolis and based on the range of bacteria tested, it seems that the propolis inhibitory spectrum is broader and its activity stronger even at very low concentrations compared to that of nisin. This should not be surprising since PEE contained a very heterogeneous collection of substances with different – and possibly synergistic – antimicrobial mode of actions while nisin has only one.

4. Conclusions

Greek and Cypriot propolis ethanolic extracts were shown to be very rich in bioactive compounds, possessing antioxidant, antibacterial and antifungal activities. Their composition presented differences from typical European propolis and similarities with East Mediterranean propolis. Despite differences in the chemical composition of propolis from different geographical locations, the PEE studied exhibited similar antibacterial and antifungal activities: they inhibited Gram positive pathogens and fungi, but did not affect several lactic acid bacteria, inhibiting in all cases a wider spectrum of microorganisms than the food grade antibiotic nisin. There is evidence that the biological action of propolis extracts is to some extend influenced by their terpene content.

Given the non-toxic and natural origin of propolis and the results obtained on their antioxidant and antimicrobial action, it is concluded that, besides their potential pharmaceutical use, low concentrations of the propolis balsams studied could be efficient protective agents for use as antioxidant and microbicidal additives in food systems, especially in fermented products, aiming to selectively inhibit the growth of pathogenic bacteria while

allowing the survival of starter culture strains like lactic acid bacteria.

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