doi: 10.17221/103/2017-CJFS

The Components Responsible for the Antimicrobial Activity of Propolis from Continental and Mediterranean Regions in Croatia

*Ivana TLAK GAJGER[1], Iva PAVLOVIĆ[2], Mirza BOJIĆ[3], Ivan KOSALEC[3], Siniša SREČEC[4], Toni VLAINIĆ[2] and Josipa VLAINIĆ[2]**

[1]Faculty of Veterinary Medicine and [3]Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia; [2]Ruđer Bošković Institute, Zagreb, Croatia; [4]Križevci College of Agriculture, Križevci, Croatia

**Corresponding author: josipa.vlainic@irb.hr*

Abstract

Tlak Gajger I., Pavlović I., Bojić M., Kosalec I., Srečec S., Vlainić T., Vlainić J. (2017): Components responsible for antimicrobial activity of propolis from continental and Mediterranean regions in Croatian. Czech J. Food Sci., 35: 376–385.

Propolis is a popular subject of research worldwide due to its therapeutic potential. The antimicrobial activity of propolis appears to be promising but depends on many variables related to its origin such as the content of phenolics and flavonoids. To address this issue with Croatian propolis, which has two major origins (Mediterranean and continental), we exposed bacteria (*Escherichia coli* and *Staphylococcus aureus*) and yeasts (*Candida albicans* and *Aspergillus niger*) to different propolis concentrations (two-fold microdilution method with TCC/formazan endpoint). Total phenolic and flavonoid content and chromatographic profile along with antioxidant activity were assessed. The majority of the 24 propolis samples tested exhibited potent antimicrobial activity against *S. aureus* bacteria and the yeast *C. albicans*. Most propolis samples also exhibited robust antioxidative capacity which correlated polyphenol and flavonoid content. To the best of our knowledge this is the first study in which the antimicrobial activity of Croatian propolis is correlated with its constituents.

Keywords: propolis; antimicrobial effects; phenols; flavonoids; DPPH; FRAP; HPLC analyses; correlations

Propolis is a sticky natural product made from a mixture of resinous substances collected by honey bees (*Apis mellifera*) from various plant sources. Because of its very complex composition, it can possess a wide range of biological activity. The propolis collected by bees fulfils many function within the honey bee hive: sealing of holes, smoothening of internal walls and protection of the entrance against intruders, as well as antimicrobial protection of the honey bee colony. Its chemical composition is highly variable depending on the collection site, floral composition and climate. More than 300 compounds related to its bioactivity and potential therapeutic use have been identified (Kujumgiev *et al.* 1999; Bankova *et al.* 2014). Its biological activity is determined mainly by compounds from the polyphenolic fraction, especially flavonoids, followed by aromatic acids, phenol acid esters, triterpenes, lignans, *etc.* (Celikel & Kavas 2008; Popova *et al*. 2014; Grenho *et al*. 2015; Machado *et al*. 2016).

The demand for the characterisation and determination of the utility of natural products, among them propolis, is increasing due to their recognised health benefits, use in the cosmetics industry and as food components (Banskota *et al*. 2001). Propolis is widely used for a variety of purposes, e.g., to relieve sore throats, to prevent and alleviate the symptoms of cold and as an antiseptic and anti-inflammatory agent. Many of the beneficial effects of propolis depend on its antimicrobial activity (Sforcin & Bankova 2011).

doi: 10.17221/103/2017-CJFS

In Croatia, the level of propolis production remains relatively low mainly due to a lack of awareness on the part of beekeepers regarding the potential beneficial properties of propolis. A further reason is its poor chemical and antimicrobial characterisation. Moreover, the diversity of continental and Mediterranean flora and the different climatic conditions in different geographical areas are additional factors influencing the biodiversity of propolis from Croatia. Thus, our aim was to assess the antimicrobial effects, antioxidant capacity and chemical constituents of propolis originating from locations throughout the territory of Croatia. The antimicrobial activity of the samples was related with total phenol and flavonoid content, as well as with the chromatographic profiles to determine if there was a pattern for the association of activity with chemical constituents. Research, such as that described here, focused on correlating the effects of propolis with its ingredients will provide a platform to characterise and evaluate the most promising candidates. Further, it will allow propolis standardisation in terms of its effects on human health.

MATERIAL AND METHODS

Sampling. Twenty-four propolis samples were taken directly from honey bee hives, and upon collection were placed into clean plastic containers, labelled and transferred to the laboratory where they were kept at room temperature until analysis. Samples originated from the continental and Mediterranean regions of Croatia, and were collected by beekeepers during the active season in 2015 (Figure 1).

Extracts preparation. Raw propolis samples (0.5 g) were mixed with 5 ml of HPLC-grade ethanol and extracted for 2 h at room temperature using an ultrasonic bath. After extraction, extracts were filtrated through Whatman Grade No. 1 Filter Paper, centrifuged at 3000 *g* and additionally filtrated using 0.45-µm membrane syringe filters. The obtained solution was adjusted with solvent up to 5 ml. All extracts were kept at 4°C in the dark and equilibrated to room temperature prior to analyses.

Extract preparation for the phytochemical analysis. Extracts of 24 different propolis samples were prepared by dissolving 0.5 g of propolis in 5 ml of 100% methanol and were stored at 4°C until analysis. Prior to analysis, extracts were sonicated for 15 min (Iskra, Croatia), vortexed and diluted 10 times in methanol (80%, v/v) for the polyphenol analysis and antioxidant activity assay.

MIC determination. The bacterial strains used in the antimicrobial susceptibility assay were *Staphylococcus aureus* ATCC 29212, *Escherichia coli* ATCC 10535, and yeasts *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC16404. The strains were sourced from stock cultures of the collection of microorganisms of Department of Microbiology,

1	Vrbovec	16°57'E, 45°92'N
2	Biograd na Moru	15°31'E, 43°59'N
3	Marčana	13°95'E, 44°95'N
4	Pag, Kolan	14°95'E, 44°49'N
5	Drabaniči	15°35'E, 45°36'N
6	Karlovac	15°74'E, 45°62'N
7	Požega	17°66'E, 46°35'N
8	Hudovo	16°32'E, 46°01'N
9	Rab	14°46'E, 44°46'N
10	Vrgovac	17°25'E, 43°24'N
11	Pazin	16°54'E, 45°92'N
12	Krapina	15°52'E, 46°09'N
13	Vis	16°11'E, 43°04'N
14	Posavski Bregi	16°22'E, 45°43'N
15	Marina	16°07'E, 43°31'N
16	Popivic	16°37'E, 45°34'N
17	Petrova Gora	15°46'E, 45°17'N
18	Čiovo	16°17'E, 43°29'N
19	Kloštar Ivanic	16°25'E, 45°44'N
20	Glina	16°16'E, 45°33'N
21	Varazdin Breg	16°31'E, 46°27'N
22	Osijek	18°40'E, 45°47'N
23	Maruševec	16°22'E, 46°27'N
24	Čemerica	17°12'E, 44°31'N

Figure 1. Map indicating the locations from where samples were collected

doi: 10.17221/103/2017-CJFS

Faculty of Pharmacy and Biochemistry at the University of Zagreb.

A serial microdilution broth assay based on the EUCAST reference documents for aerobic bacteria was used to determine minimum inhibitory concentrations (MIC) (EUCAST 2003). Briefly, cell suspensions of bacteria were prepared from stock cultures (kept at –30°C in 25% glycerol-nutrient broth) and maintained on a surface of trypticase soy agar for 18 h at 37°C. Inoculums were prepared using physiological saline and adjusted using a nephelometer (ATB 1550; BioMérieux, France) to 0.5 MacFarland units. Microdilution was performed in Mueller-Hinton broth with serially diluted ethanolic extracts of propolis ranging from 25% to 0.098% in microtiter plates. After incubation, MICs, recorded as the lowest concentrations resulting in 100% growth inhibition of propolis extracts, were determined after re-inoculation of each dilution on the surface of trypticase soy agar and incubation for 18 h at 37°C.

Polyphenol analysis. The phytochemical content of propolis extracts was determined spectrophotometrically. Additional dilutions of extracts were performed to fit the samples to the standard curves. The total polyphenol content was measured using the Folin-Ciocalteu reagent (Singleton & Rossi 1965) with gallic acid as a standard, and results were expressed per g of lyophilised powder (mg GAE/g). Total flavonoid content was measured using an $AlCl_3$ colorimetric assay (Zhisten *et al*. 1999) adapted to small volumes (Šamec *et al*. 2011). Mixing of the propolis extract with water results in a cloudy solution so after the reactions were finished an additional centrifugation step was introduced to eliminate any particles. Catechin was used as a standard and results were expressed per g of lyophilised powder (mg CE/g).

HPLC analysis of flavonoids and phenolic acids. HPLC analysis of phenolic acids and flavonoid aglycones was performed on an Agilent 1100 instrument equipped with a diode array detector (Medić-Šarić *et al*. 2011). Samples were diluted in a 1 : 49 ratio with ethanol. Separation was achieved on a Zorbax SB-C18 (250 mm × 4.6 mm, particle size 5 µm) with a precolumn (12.5 mm × 4.6 mm, particle size 5 µm) using a mobile gradient of mobile phases A (water, methanol and formic acid in a 93 : 5 : 2 ratio) and B (water, methanol and formic acid in a 3 : 95 : 2 ratio) with the following timetable (*t*/min, %B): (0, 20), (10, 40), (35, 50), (47, 50), (70, 80), and (80, 20). Detection was based on retention times and UV spectra compared to the standards. Quantification was based on calibration curves recorded at 270 (chrysin, tectochrysin), 290 (pinocembrin, pinocembrin-7-methylether), 320 (apigenin, ferulic and *p*-coumaric acids), and 350 nm (galangin, kaempferol).

Antioxidant activity of propolis samples. DPPH and the FRAP assay were utilised to determine the antioxidant potential of propolis extracts. The radical scavenging capacity of extracts against the DPPH radical was tested (Brand-Williams *et al*. 1995). A standard curve was constructed using Trolox and the results were expressed as µmol Trolox per g of lyophilised powder (µmol Trolox/g). The ferric reducing/antioxidant power of extracts was determined (Benzie & Strain 1999) and results are expressed as µmol of $FeSO_4 \cdot 7H_20$/g of lyophilised powder (µmol Fe^{2+}/g).

Statistical analysis. The experiments were performed in triplicate on three independent occasions. Mean and standard deviation values are presented. Statistical analyses were performed using GraphPad

Table 1. Minimal inhibitory concentrations (MIC) of propolis samples

	S. aureus ATCC 6538P	*E. coli* ATCC10536	*C. albicans* ATCC 10231	*A. niger* ATCC16404
1	6.25	> 50	6.25	> 50
2	1.56	> 50	1.56	6.25
3	> 50	> 50	> 50	> 50
4	3.12	> 50	3.12	> 50
5	12.50	> 50	6.25	> 50
6	3.12	> 50	3.12	> 50
7	1.56	> 50	3.12	> 50
8	12.50	> 50	6.25	> 50
9	12.50	> 50	6.25	> 50
10	6.25	> 50	3.12	> 50
11	12.50	> 50	1.56	> 50
12	0.78	12.50	0.78	12.50
13	1.56	> 50	6.25	> 50
14	0.78	> 50	1.56	> 50
15	12.50	> 50	> 50	> 50
16	0.78	> 50	1.56	12.50
17	> 50	> 50	6.25	> 50
18	> 50	> 50	6.25	> 50
19	3.12	> 50	3.12	> 50
20	12.50	> 50	3.12	> 50
21	1.56	> 50	6.25	> 50
22	> 50	> 50	> 50	> 50
23	0.39	> 50	1.56	12.50
24	0.39	> 50	1.56	12.50

doi: 10.17221/103/2017-CJFS

Prism 4.0 software and $P < 0.05$ was considered statistically significant. The Spearman rank-order correlation was assessed using the statistical software SPSS Statistics (IBM, USA).

RESULTS

Minimal inhibitory concentrations of propolis samples. To reveal the antimicrobial potency of the collected Croatian propolis samples we used an antimicrobial susceptibility assay. The results showed that no samples exhibited activity against the Gram-negative microorganism *E. coli*, while many were effective against *A. niger* (except samples 2, 12, 16, 23, and 24). These samples had MIC values of 6.25 and 12.5 mg/ml, respectively. Although several samples had MIC values of above 50 mg/ml for the Gram-positive bacterium *S. aureus* (samples 3, 17, 18, and 22) and yeast *C. albicans* (3, 15, and 22), other samples had MIC values within the range (0.391–12.5 mg/ml) (Table 1) which is considered indicate potential for therapeutic purposes.

Polyphenol analysis of propolis samples. Most of the samples presented total polyphenol values in the range of 70–220 g GAE/kg, although three propolis

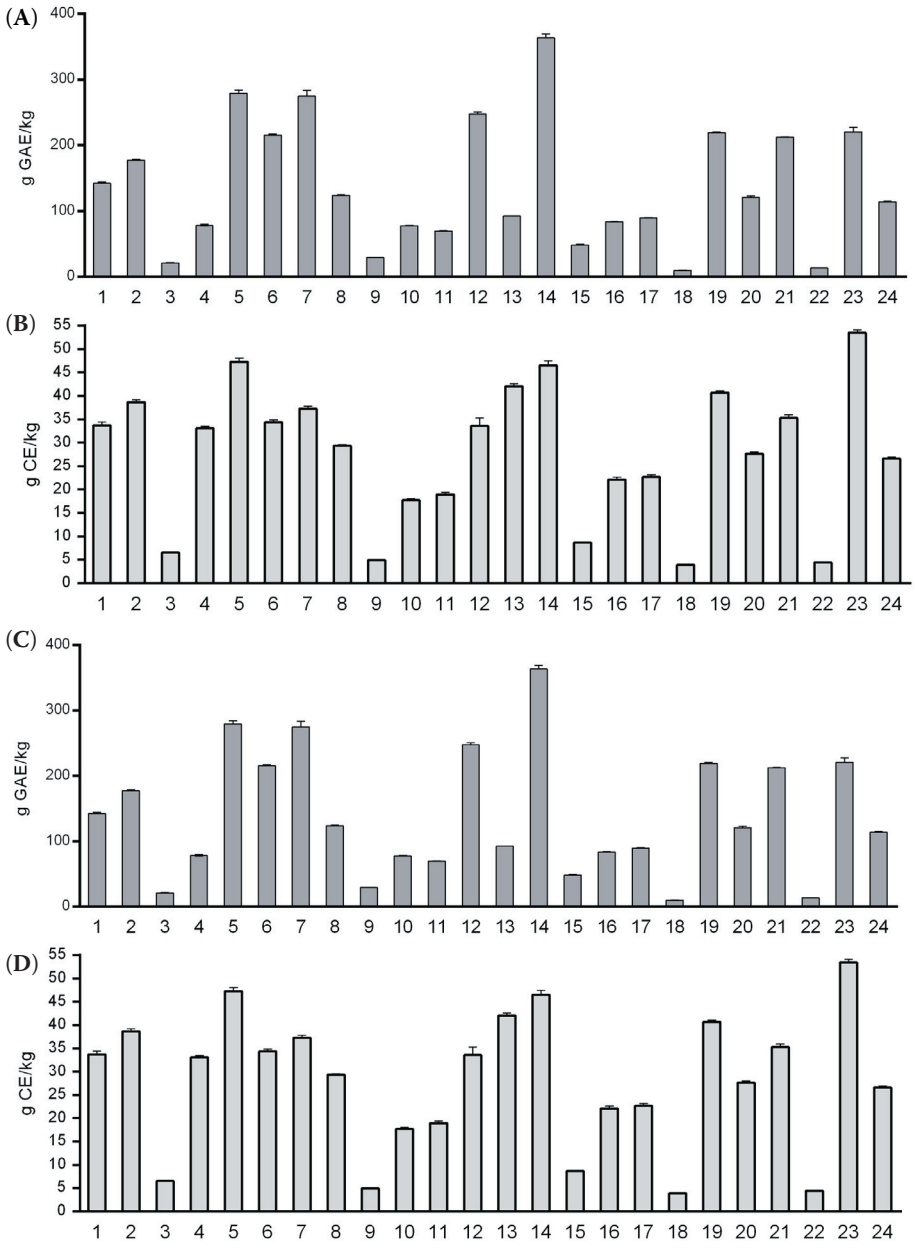

Figure 2. Determination of total phenols (g GAE/kg), flavonoids (g CE/kg), and antioxidative capacity (DPPH – mmol Trolox/kg and FRAP – mmol Fe/kg)

Table 2. Content of individual polyphenols in analysed propolis samples

Sample	Ferrulic acid	*p*-Coumaric acid	Tectochrysin	Galangin	Pinocembrin	Pinocembrin-7-metylether	Chrysin	Apigenin	Kaempferol
1	0.685 ± 0.008	0.582 ± 0.006	0.79 ± 0.04	0.341 ± 0.009	0.220 ± 0.010	nd	0.350 ± 0.020	0.070 ± 0.010	nd
2	0.032 ± 0.001	0.136 ± 0.008	2.60 ± 0.20	0.460 ± 0.020	0.840 ± 0.010	0.240 ± 0.004	1.000 ± 0.010	0.220 ± 0.010	0.133 ± 0.0089
3	nd	nd	0.62 ± 0.04	nd	3.400 ± 0.010	0.036 ± 0.001	0.064 ± 0.002	nd	nd
4	0.232 ± 0.004	0.424 ± 0.004	10.96 ± 0.09	0.594 ± 0.009	0.802 ± 0.009	0.576 ± 0.002	3.700 ± 0.030	0.145 ± 0.008	0.100 ± 0.009
5	1.370 ± 0.020	1.020 ± 0.010	1.19 ± 0.07	0.250 ± 0.030	0.523 ± 0.009	0.045 ± 0.002	0.298 ± 0.006	0.099 ± 0.003	0.126 ± 0.009
6	0.714 ± 0.006	0.864 ± 0.001	1.19 ± 0.03	0.460 ± 0.020	0.407 ± 0.003	0.048 ± 0.002	0.650 ± 0.010	0.103 ± 0.008	0.087 ± 0.005
7	0.063 ± 0.001	0.523 ± 0.005	2.46 ± 0.03	0.750 ± 0.020	1.430 ± 0.010	0.139 ± 0.005	1.530 ± 0.030	0.110 ± 0.010	0.139 ± 0.005
8	0.796 ± 0.004	1.031 ± 0.006	0.27 ± 0.01	0.298 ± 0.009	0.075 ± 0.002	0.063 ± 0.001	0.073 ± 0.001	0.071 ± 0.003	nd
9	nd	0.017 ± 0.001	0.18 ± 0.01	nd	0.066 ± 0.002	0.039 ± 0.001	0.143 ± 0.002	0.064 ± 0.006	nd
10	nd	nd	nd	nd	0.023 ± 0.001	nd	nd	nd	nd
11	0.618 ± 0.007	0.540 ± 0.030	0.62 ± 0.01	0.174 ± 0.004	0.097 ± 0.002	0.048 ± 0.002	0.228 ± 0.002	0.060 ± 0.003	0.119 ± 0.005
12	0.297 ± 0.001	0.363 ± 0.001	16.07 ± 0.05	8.710 ± 0.020	6.390 ± 0.003	0.423 ± 0.002	8.020 ± 0.010	1.230 ± 0.010	0.672 ± 0.009
13	0.047 ± 0.001	0.787 ± 0.001	4.66 ± 0.03	0.372 ± 0.004	0.622 ± 0.008	0.267 ± 0.004	nd	0.169 ± 0.006	0.264 ± 0.005
14	0.693 ± 0.001	0.731 ± 0.002	13.59 ± 0.05	nd	nd	0.113 ± 0.001	nd	0.430 ± 0.020	0.370 ± 0.010
15	0.027 ± 0.001	0.006 ± 0.001	0.39 ± 0.01	0.168 ± 0.004	0.114 ± 0.002	nd	0.162 ± 0.002	0.395 ± 0.006	0.119 ± 0.005
16	0.091 ± 0.001	0.182 ± 0.001	3.19 ± 0.07	2.630 ± 0.020	2.683 ± 0.002	0.155 ± 0.002	2.730 ± 0.010	0.239 ± 0.003	0.212 ± 0.005
17	0.772 ± 0.001	0.703 ± 0.001	0.75 ± 0.01	0.341 ± 0.004	0.144 ± 0.002	nd	0.142 ± 0.002	0.099 ± 0.006	0.093 ± 0.005
18	0.021 ± 0.001	nd	nd	nd	0.032 ± 0.001	nd	nd	nd	nd
19	1.020 ± 0.002	0.730 ± 0.004	2.86 ± 0.03	0.800 ± 0.020	0.996 ± 0.004	0.054 ± 0.001	1.590 ± 0.010	0.118 ± 0.003	0.126 ± 0.005
20	0.627 ± 0.001	0.772 ± 0.001	0.33 ± 0.01	0.162 ± 0.004	0.100 ± 0.002	nd	0.057 ± 0.005	0.071 ± 0.003	nd
21	0.785 ± 0.004	0.822 ± 0.001	1.45 ± 0.01	0.588 ± 0.004	0.922 ± 0.005	0.061 ± 0.001	0.678 ± 0.001	0.640 ± 0.010	nd
22	0.024 ± 0.001	nd	nd	nd	0.050 ± 0.001	nd	0.074 ± 0.004	nd	nd
23	0.392 ± 0.001	0.772 ± 0.001	5.25 ± 0.03	2.360 ± 0.020	2.200 ± 0.050	0.171 ± 0.002	2.680 ± 0.040	0.290 ± 0.003	0.245 ± 0.009
24	0.175 ± 0.002	0.198 ± 0.002	8.30 ± 0.04	2.190 ± 0.010	2.510 ± 0.006	0.070 ± 0.002	2.000 ± 0.010	0.149 ± 0.003	0.192 ± 0.005

Czech J. Food Sci., 35, 2017 (5): 376–385

doi: 10.17221/103/2017-CJFS

doi: 10.17221/103/2017-CJFS

samples had significantly lower polyphenolic content (Figure 2). These were samples 3 and 9 which had 21.09 and 29.42 g GAE/kg, respectively, and sample 15 with 48.33 g GAE/kg. Relatively low levels of polyphenols were obtained in samples 18 (9.67 g GAE/kg) and 22 (13.36 g GAE/kg). A large variability in total flavonoid content was also observed in most samples, ranging from 30 to 50 g CE/kg. Moreover, a similar distribution pattern as for total polyphenols was observed for flavonoid content, i.e., samples 3, 9, 15, 18, and 22 had relatively low levels of polyphenols (appr. 5 g CE/kg) (Figure 2).

HPLC analysis: propolis fingerprint. The content of individual polyphenols in the 24 analysed propolis samples is shown in Table 2. The most widespread polyphenol across all propolis samples was kaempferol, which was detected in 23 out of 24 samples. Techtochrysin was the most abundant flavonoid aglycone with content of up to 16.07 mg per ml of extract. Sample number 12 showed the highest content of individual flavonoids, namely, tectochrysin (16.07 mg/ml), galangin (8.71 mg/ml), pinocembrin (6.39 mg/ml), chrysin (8.02 mg/ml), apigenin (1.23 mg/ml) and kampferol (0.67 mg/ml). Content of ferrulic and *p*-coumaric acid was 1.37 and 1.03 mg/ml, respectively.

Antioxidant activity and scavenging capacity of propolis samples. The most active samples in the DPPH assay, with values ranging from 960–1200 mmol Trolox/kg, were samples 2 (964.53 mmol Trolox/kg), 5 (983.28 mmol Trolox/kg), 7 (1006.34 mmol Trolox/kg), and 14 (1198.10 mmol Trolox/kg); most of the samples varied between 140 and 640 mmol Trolox/kg. Less active were samples 3 and 9 with activities of 60 mmol Trolox/kg while samples 18 and 22 had very low levels of DPPH activity (30.93 and 28.40 mmol Trolox/kg, respectively) when compared to others (Figure 2).

The most potent scavenging activity was exhibited by samples 1 (900.0 mmol Fe^{2+}/kg), 2 (1210.0 mmol Fe^{2+}/kg), 5 (1337.22 mmol Fe^{2+}/kg), 6 (1012.22 mmol Fe^{2+}/kg), 7 (1283.89 mmol Fe^{2+}/kg), 12 (1092.78 mmol Fe^{2+}/kg), 19 (1117.78 mmol Fe^{2+}/kg), and 21 (1085.0 mmol Fe^{2+}/kg). The samples with the lowest activity were samples 3 (75.61 mmol Fe^{2+}/kg), 18 and 22 (approx. 40 mmol Fe^{2+}/kg) (Figure 2).

DISCUSSION

Extracts from 24 propolis samples collected from both the continental and Mediterranean regions of Croatia were assayed for antimicrobial potency against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria of clinical importance. The bacterial strains were chosen due to the fact that propolis is used to treat skin infections topically and is also used as a remedy in gastrointestinal infections. Moreover, in our research we also included yeast *C. albicans* (opportunistic oral and genital infections) and *A. niger* (fungal ear infection) which cause infections accessible for topical treatment. The results are presented as MIC values (Table 1), except for *E. coli* since no antibacterial activity could be detected and all MIC values were above 50 mg/ml. Nina *et al.* (2015) described limited activity of propolis samples from Argentina against *E. coli*, while others (Boyanova *et al*. 2006; Kosalec *et al*. 2007) found no inhibitory activity of Bulgarian and Brazilian propolis, respectively, against *E. coli*. Kosalec *et al.* (2007) found MIC values of below 10.4 mg/ml against *E. coli* while testing Croatian propolis samples. On the other hand, it has been shown that Brazilian and Korean propolis inhibit the Gram-negative bacterium *S. typhimurium*, but have no activity against *P. aeruginosa* (Choi *et al*. 2006). Efficient antibacterial action of propolis against *S. aureus* has been shown previously (Kujumgiev *et al*. 1999; Drago *et al*. 2000; Sforcin *et al*. 2000), while *E. coli* was more described to be resistant to propolis activity (Drago *et al*. 2000; Sforcin *et al*. 2000).

It is interesting to note that some propolis extracts were active against *A. niger*. These extracts were those with the highest overall activity against the tested microorganisms and had lower MIC values than the other samples. The mentioned samples, except for sample 2 (coastal region), were collected in the northern part of Croatia (Figure 1) and may be considered to be of similar geographical and floral origin. Samples 17 and 18 showed considerable activity against *C. albicans*. Antifungal effects have been described previously (Kujumgiev *et al*. 1999; Moreno *et al*. 1999; Sawaya *et al*. 2002), although studies have also shown that propolis may have no anti-candidiasis effect (Nina *et al*. 2015). In our study, there were large differences in anti-candidiasis efficacy, indicating differences in origin as a source of variability in the sample activity, an idea which is further supported by the observation that samples from the same region had similar antifungal properties.

Samples 3 and 22 showed poor antimicrobial activity with MICs for all microorganisms tested

doi: 10.17221/103/2017-CJFS

> 50 mg/ml. However, the samples were geographically very different: one originated from Istria (inner part) and the other from continental Croatia. These two regions are quite different in climate and geographical characteristics (Figure 1), and the samples have different botanical and floral origin. The highest MIC values were also obtained for samples 15, 17, and 18. It is interesting to note that one sample with the most potent antimicrobial effect (16) and one sample which was least active (22) were collected within the same area.

Most of our propolis samples presented total polyphenol values in the range of 70–220 g GAE/kg (Figure 2). A large variability in total flavonoid content was also observed. Compared to previous analysis of Croatian propolis (Barbarić *et al.* 2011), flavonoids were detected in a greater number of samples at higher levels. This can be attributed to differences in the extraction procedure, as we utilised an ultrasound-facilitated extraction procedure that results in greater extraction yields and more efficient liberation of lipophilic flavonoids from waxy propolis matrices (Trusheva *et al.* 2007).

The results showed that samples with pronounced DPPH activity were most active in the FRAP assay, and those with relatively low antioxidative capacity exhibited low activity in both assays (Figure 2). The variability in the antioxidant activity can be attributed to the composition of the samples, which is related to the botanical sources of propolis. Flavonoids and phenolic acid derivatives may be responsible for the antioxidant activity. Indeed, significant correlations between total phenol content and antiradical activity ($r = 0.92$, $P < 0.05$) and between total phenolic content and scavenging activity were found ($r = 0.94$, $P < 0.05$). Moreover, the total flavonoid content significantly correlated with antiradical activity ($r = 0.85$, $P < 0.05$) and reducing power ($r = 0.86$, $P < 0.05$) (Table 3). Taken together, the Spearman rank-order correlations between total phenol and flavonoid and DPPH and FRAP indicated that phenolic compounds have a more important role than flavonoids in determining scavenging capacity and antioxidant power. Similar observations have been made with Turkish propolis: propolis with the greatest phenol and flavonoid content exhibited the most pronounced antioxidant activity (Popova *et al.* 2005; Živković *et al.* 2010). Thus, our results support the hypothesis of a high correlation between the antioxidant activity and phenolics and flavonoids.

The antioxidant activity of propolis is well-recognised and is considered to be one of the potential mechanisms of its beneficial effects. Thus, the correlation of MIC values for *S. aureus* and *C. albicans* and the content of total phenols and flavonoids is highly significant as is the correlation between antioxidant activity/scavenging capacity and the antimicrobial potency of the samples (Figure 2). The involvement of flavonoid chemistry in the biological activities of propolis, such as antioxidant and antimicrobial properties, has been shown for, among others, Spanish propolis (Bonhevi & Gutierrez 2012). By scavenging reactive oxygen and nitrogen species, propolis may interfere with potentially harmful processes within organisms and may even interrupt the reactions that could lead to lipid peroxidation. The chelation of metal ions (in our study ferric ions), which has been shown to occur at considerable levels, is also one of the desirable activities of propolis, since these substances can lead to cellular damage.

The correlation studies showed highly significant correlations between antimicrobial activity and antioxidative capacity (Table 3). Taken together with the significant correlation of polyphenol and flavonoid content with antimicrobial effects, this indicates that propolis should be selected for commercial use based on its polyphenol and flavonoid contents. Namely, it has been shown that flavonoids and phenolic acid derivatives may be responsible for its antioxidant activity.

A high correlation between the total phenolic and flavonoid content and free radical scavenging activity was also reported for propolis samples from other countries; e.g., Argentina (Vera *et al.* 2011), Japan (Hamasaka *et al.* 2004), Greece and Cyprus

Table 3. Spearman Rank Order Correlations between propolis polyphenol and flavonoid content and its antimicrobial and antioxidative efficacy

	MIC *S. aureus*	MIC *C. albicans*	DPPH	FRAP
Total polyphenols	–0.58*	–0.47*	0.92*	0.94*
Flavonoid content	–0.67*	–0.59*	0.85*	0.86*

*$P < 0.05$

doi: 10.17221/103/2017-CJFS

Table 4. Correlations between chemical compounds of propolis (independent variables) and antioxidant capacity (DPPH and FRAP) and antimicrobial potential (MIC)

	DPPH	FRAP	MIC	
			S. aureus	*C. albicans*
Ferrulic acid	0.42*	0.59*	0.55	0.43
p-Coumaric acid	0.55*	0.67*	0.50	0.62*
Tectochrysin	0.41*	0.34	–0.68*	–0.45
Galangin	0.12	0.20	–0.83*	–0.53
Pinocembrin	0.04	0.10	–0.86*	–0.57
Pinocembrin-7-metylether	0.26	0.22	–0.54	–0.48
Chrysin	0.14	0.22	–0.65*	–0.42
Apigenin	0.30	0.38	–0.84*	–0.68*
Kaempferol	0.42*	0.39	–0.82*	–0.67*

*$P < 0.05$

(Kalogeropoulos *et al*. 2009), Brazil (da Silva *et al*. 2006), Turkey (Popova *et al*. 2005), and Poland (Socha *et al*. 2015).

Correlations between individual compounds and antioxidant and antimicrobial activity were also analysed (Table 4). Concentrations of ferulic acid, *p*-coumaric acid, tectochrysin, and kaempferol showed significant correlations with DPPH, while only ferulic and p-coumaric acid showed significant correlations with ferric reducing activity ($P < 0.05$). Galangin, pinocembrin, chrysin, and apigenin had no correlation with antioxidant activities. On the other hand, the content of tectochrysin, galangin, pinocembrin chrysin, apigenin, and kaempferol was significatly correlated with antimicrobial activity while *p*-coumaric acid, apigenin, and kaempferol were significantly correlated with the activity of propolis against *C. albicans*. The high concentrations of flavonoids and aromatic acids such as galangin, kaempferol, pinostrobin, and pinocembrin are responsible, at least partially, for the antibacterial effects of propolis (Wang *et al*. 2016). It has been shown that flavonoids, and specifically kaempferide, quercetin, galangin, and pinocembrin, interfere with bacterial RNA polymerase and cause its inhibition. One other mechanism proposed for the antibacterial activity of flavonoids, caffeic, benzoic and 4-hydroxy-3,5-diprenylcinnamic acids is of cell wall and causing structural binding to microbial membranes or cell walls, in this way causing structural and functional damage. It has been shown that chemical components of propolis may harbour dose-dependent cytocidal activity and an inhibitory effect on yeast-mycelial conversion, and that they may inhibit extracellular phospholipase activity and fungal adhesion to epithelial cells (D'Auria *et al*. 2003).

CONCLUSION

This is the first in-depth study of Croatian propolis in terms of antimicrobial activity and the correlation of different compounds with antimicrobial and antioxidant properties. The data suggest that Croatian propolis samples have varying antimicrobial potencies, which further indicates that individual compounds might make different contributions to the total antioxidant and/or antimicrobial activity of propolis of the poplar type. Additional research is needed to determine whether synergistic or additive effects may exist among individual propolis components in their antioxidant and antimicrobial activities. Moreover, our results suggest that it is important to determine the type of propolis with respect to its bioactivity, and that evaluation and establishment of criteria for standardization of propolis quality are needed.

References

Bankova V., Popova M., Trusheva B. (2014): Propolis volatile compounds: chemical diversity and biological activity: a review. Chemistry Central Journal, 8: 28. doi: 10.1186/1752-153X-8-28

Banskota A.H., Tezuka Y., Kadota S. (2001): Recent progress in pharmacological research of propolis. Phytotheraphy Research, 15: 561–571.

Barbarić M., Mišković K., Bojić M., Lončar M.B., Smolčić-Bubalo A., Debeljak Z., Medić-Šarić M. (2011): Chemical composition of the ethanolic propolis extracts and its effect on HeLa cells. Journal of Ethnopharmacology, 135: 772–778.

Benzie I.F., Strain J.J. (1999): Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymology, 299: 15–27.

Bonheví J.S., Gutiérrez A.L. (2012): The antimicrobial effects of propolis collected in different regions in the Basque country (Northern Spain). World Journal of Microbiology and Biotechnology, 28: 1351–1358.

Boyanova L., Kolarov R., Gergova G., Mitov I. (2006): *In vitro* activity of Bulgarian propolis against 94 clinical isolates of anaerobic bacteria. Anaerobe, 12: 173–177.

doi: 10.17221/103/2017-CJFS

Brand-Williams W., Cuvelier M.E., Berset C. (1995) Use of a free radical method to evaluate antioxidant activity. LWT- Food Science and Technology, 28: 25–30.

Celikel N., Kavas G. (2008): Antimicrobial properties of some essential oils against some pathogenic microorganisms. Czech Journal of Food Sciences, 26: 174–181.

Choi Y.M., Noh D.O., Cho S.Y., Suh H.J., Kim K.M., Kim J.M. (2006): Antioxidant and antimicrobial activities of propolis from several regions of Korea. LWT-Food Science and Technology, 39: 756–761.

da Silva J.F.M., de Souza M.C., Matta S.R., de Andrade M.R., Vidal F.V.N. (2006): Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. Food Chemistry, 99: 431–435.

D'Auria F.D., Tecca M., Scazzocchio F., Renzini V., Strippoli V. (2003): Effect of propolis on virulence factors of *Candida albicans*. Journal of Chemotherapy, 15: 454–460.

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 Chemotheraphy, 12: 390–395.

EUCAST Discussion Document E. Def 2003, 5.1. (2003): European Committee on Antimicrobial Susceptibility Testing (EUCAST). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth microdilution. Clinical Microbiology and Infection, 9: 1–10. Available at (http://www.escmid.org/Seviware/Script/SvFiles.asp?Ref=359)

Grenho L., Barros J., Ferreira C., Santos V.R., Monteiro F.J., Ferraz M.P., Cortes M.E. (2015): *In vitro* antimicrobial activity and biocompatibility of propolis containing nanohydroxyapatite. Biomedical Materials, 10 (2): 025004. doi: 10.1088/1748-6041/10/2/025004

Hamasaka T., Kumazawa S., Fujimoto T., Nakayama T. (2004): Antioxidant activity and constituents of propolis collected in various areas of Japan. Food Science and Technology Research, 10: 86–92.

Kalogeropoulos N., Konteles S.J., Troullidou E., Mourtzinos I., Karathanos V. (2009): Chemical composition, antioxidant activity and antimicrobial properties of propolis extract from Greece and Cyprus. Food Chemistry, 116: 452–461.

Kosalec I., Sankovic K., Zovko M., Bakmaz M., Kalogjera Z., Pepljnjak S. (2007): Antimicrobial and antioxidant activity of propolis from Croatia and Brazil: a comparative study. In: Bauer R. (ed.): Planta Medica: Book of abstracts 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Sept 2–6, 2007, Graz, Austria.

Kujumgiev A., Tsvetkova I., Serkedjieva Yu., Bankova V., Christov R., Popov S. (1999): Antibacterial, antifungal and antiviral activity of propolis from different geographic origins. Journal of Ethnopharmacology, 64: 235–240.

Machado C.S., Mokochinski J.B., de Lira T.O., de Oliveira F.D.C.E., Cardoso M.V., Ferreira R.G., Sawaya A.C., Ferreira A.G., Pessoa C., Cuesta-Rubio O., Monteiro M.C., de Campos M.S., Torres Y.R. (2016): Comparative study of chemical composition and biological activity of yellow, green, brown, and red Brazilian propolis. Evidence-Based Complementary and Alternative Medicine, 2016, Article ID 6057650. doi: 10.1155/2016/6057650

Medić-Sarić M., Rastija V., Bojić M. (2011): Recent advances in the application of high performance liquid chromatography in the analysis of polyphenols in wine and propolis. Journal of AOAC International, 94: 32–42.

Moreno M.I.N., Isla M.I., Cudmani N.G., Vattuone M.A., Sampietro A.R. (1999): Screening of antibacterial activity of Amaicha del Valle (Tucumán, Argentina) propolis. Journal of Ethnopharmacology, 68: 97–102.

Nina N., Quispe C., Jiménez-Aspee F., Theoduloz C., Feresín G.E., Lima B., Leiva E., Schmeda-Hirschmann G. (2015): Antibacterial activity, antioxidant effect and chemical composition of propolis from the Región del Maule, Central Chile. Molecules, 20: 18144–18167.

Popova M., Silici S., Kaftanoglu O., Bankova V. (2005): Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. Phytomedicine, 12: 221–228.

Šamec D., Piljac-Žegarac J., Bogović M., Habjanič K., Grúz J. (2011): Antioxidant potency of white (*Brassica oleracea* L. var. *capitata*) and Chinese (*Brassica rapa* L. var. *pekinensisi* (Lour.)) cabbage: The influence of development stage, cultivar choice and seed selection. Scientia Horticulturae. 128: 78–83.

Sawaya A.C., Palma A.M., Caetano F.M., Marcucci M.C., Cunha I.B.S., Araujo C.E. (2002): Comparative study of in vitro methods used to analyse the activity of propolis extracts with different compositions against species of Candida. Letters in Applied Microbiology, 35: 203–207.

Sforcin J.M., Fernandes A. Jr., Lopes C.A., Bankova V., Funari S.R. (2000): Seasonal effect on Brazilian propolis antibacterial activity. Journal of Ethnopharmacology, 73: 243–249.

Sforcin J.M., Bankova V. (2011): Propolis: is there a potential for the development of new drugs? Journal of Ethnopharmacology, 133: 253–260.

Singleton V.L., Rossi J.A. (1965): Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16: 144–158.

Socha R., Gałkowska D., Bugaj M., Juszczak L. (2015): Phenolic composition and antioxidant activity of propolis

doi: 10.17221/103/2017-CJFS

from various regions of Poland. National Product Research, 29: 416–422.

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

Vera N., Solorzano E., Ordoñez R., Maldonado L., Bedascarrasbure E., Isla M.I. (2011): Chemical composition of Argentinean propolis collected in extreme regions and its relation with antimicrobial and antioxidant activities. National Product Communications, 6: 823–827.

Wang X., Sankarapandian K., Cheng Y., Woo S.O., Kwon H.W., Perumalsamy H., Ahn Y.J. (2016): Relationship between total phenolic contents and biological properties of propolis from 20 different regions in South Korea. BMC Complementary and Alternative Medicine, 16: 65. doi: 10.1186/s12906-016-1043-y

Zhisten J., Mengcheng T., Jiamming W. (1999): The determination of flavonoid contents in mulberry and their scavenging effect on superoxide radicals. Food Chemistry, 64: 555–559.

Živković J., Zeković Z., Mujić I., Vidović S., Cvetković D., Lepojević Ž., Nikolić G., Trutić N. (2010): Scavenging capacity of superoxide radical and screening of antimicrobial activity of *Castanea sativa* Mill. extracts. Czech Journal of Food Sciences, 28: 61–68.

Received: 2017–03–08
Accepted after corrections: 2017–08–28
Published online: 2017–09–13