Original Article

COMPARATIVE STUDY ON PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF PROPOLIS COLLECTED FROM DIFFERENT REGIONS OF BULGARIA

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Received: 22 December 2022; accepted: 02 May 2023

Abstract

Propolis (bee glue), a valuable biological mixture produced by European honey bees (*Apis mellifera* L.), is widely used as a remedy in traditional and alternative medicine, as an effective food biopreservative and as a nutritional value enhancer. The present study aimed to investigate eighty propolis samples collected from all twenty-eight districts of Bulgaria in the period of 2020-2022 in order to determine their physicochemical properties as well as their antioxidant and antimicrobial potential. The raw propolis samples exhibited brown or green colour depending on the climatic zone from which they are collected and pH values from 4.82 to 5.87, while the moisture content ranged between 0.98% and 2.97%. The total phenolics content (TPC) and total flavonoids content (TFC) varied from 63.14 mg GAE/g to 737.27 mg GAE/g, and from 29.22 mg QE/g to 234.17 mg QE/g, respectively. The propolis samples demonstrated significant antioxidant potential determined by DPPH and FRAP methods, from 18.56 mM TE/g to 1598.66 mM TE/g and from 82.28 mM TE/g to 1208.81 mM TE/g, respectively, whose values showed a positive correlation (r^2) with the TPC and TFC. The results from the antimicrobial screening revealed that the methanolic propolis extracts in concentration of 20 mg/ml exhibited the highest inhibitory effect on the Gram-positive bacteria such as *Micrococcus luteus* 2YC-YT, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632 and *Listeria innocua* ATCC 33090.

Keywords: antimicrobial activity, antioxidant activity, Bulgaria, physicochemical properties, propolis

INTRODUCTION

Propolis (bee glue) is a complex biological mixture produced by European honey bees (*Apis mellifera* L.) after collecting exudates from flowers and leaf buds of various plant species. Propolis plays an important role as a building and defensive material that bees use to fill up cracks, smooth the internal hive walls, repair and seal up the honeycomb cells, and embalm the corpses of invaders who have penetrated and died inside the hive (Sforcin, 2007; Wagh, 2013; Topuzova et al., 2021). In order to produce propolis, the worker bees transport the plant material to the hive and mix it with beeswax and saliva secreted from their salivary glands, thereby obtaining a substance with a sticky consistency. Crude propolis is a hard wax-like substance when cool, but soft, plastic and sticky when heated to 36°C (De Groot, 2013).

As of 2018, the number of the chemical compounds identified as constituents of propolis has reached 850 (Bouchelaghem, 2022). Among the major chemical components are polyphenols (mainly flavonoids), followed by aromatic acids and esters, aliphatic acids and esters, volatile compounds, hydrocarbons, steroids, enzymes, waxy acids, alcohols, aldehydes, ketones, amino acids, micro- and macronutrients, vitamins, essential oils, sugars, pollen and other organic matter (Sahinler & Kaftanoglu, 2005; Toreti et al., 2013; Huang et al., 2014; Bouchelaghem, 2022). The colour (green, brown, red or yellow), the chemical composition and biological properties of propolis are highly variable depending on the plant source and the characteristics of the geographic region from which it is collected (Bankova et al., 2016).

According to the influence of certain climatic factors, Bulgaria is divided into five climatic zones (temperate continental, transitional continental, continental Mediterranean, Black Sea and mountain), which are characterized by a great botanical diversity. The main sources of propolis are the bud exudates from *Populus* spp. (*Populus nigra* L. and *P. tremula* L.), pine (*Pinus* L.), alder (*Alnus glutinosa* L.), horse chestnut (*Aesculus hippocastanum* L.), elm (*Ulmus laevis* Pallas, *U. minor* Mill and *U. glabra* Huds.), ash (*Fraxinus excelsior* L.), oak (*Quercus* spp. L.), beech (*Fagus sylvatica* L.), birch (*Betula verrucosa* Ehrh.), white willow (*Salix alba* L.), cherry (*Prunus padus* L.) and sour cherry (*P. cerasus* Gray). Therefore, the major biogically active compounds in poplar-type (Eurasian) propolis are the chemical compounds typical of these tree species (dihydrocaffeic acid, dihydroferulic acid, pinostrobin, dimethyl kaempferol, benzyl alcohol, pinobanksin, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin, myricetin, kaempferol, rutin, catechin, quercetin-3-β-glucoside, alcohols, aromatic acids, organic acids and terpenoids) (Bankova, 2005; Ristivojević et al., 2015; Dezmirean et al., 2021; Topuzova et al., 2021; Bouchelaghem, 2022). The propolis from Russia, Belarus, Ukraine and Poland, usually collected from white and silver birch (*Betula pendula* Roth), is different in comparison with the poplar-type propolis, and comprises mainly flavonols and flavones (Ristivojević et al., 2015). The red propolis from Brazil, Cuba, Mexico, Venezuela and China, originating from plant species as *Dalbergia ecastaphyllum* (L.) Taub., *Clusia scrobiculata* Benoist, *C. minor* L., *C. major* L. and *C. rosea* Jacq., is characterized by polyisoprenylated benzophenones as main active phytocomponents. The Brazilian brown propolis, obtained from *Baccharis dracunculifolia* DC., contains a variety of phytochemicals, including flavonoids, lignans, p-coumaric acid, diterpenes, acetophenone and artepillin C (Boudourova-Krasteva et al., 1997; Bankova et al., 1998; Moise & Bobiş, 2020). The propolis from the Mediterranean region (Bulgaria, Greece, Italy, Cyprus, Croatia, Malta, Turkey, Egypt, Algeria and Morocco), originating from coniferous plants of the family *Cupressaceae*, contains mainly flavonoids and esters of caffeic and ferulic acids but also significant amounts of diterpenes as active phytochemical compounds, especially in Algerian propolis (Velikova et al., 2000; Popova et al., 2011).

The complex chemical composition of propolis determines its wide spectrum of biological and pharmacological properties. Propolis has been found to demonstrate such beneficial health effects as antibacterial, antiviral, antiparasitic, antioxidant, immunomodulatory, anti-inflammatory, anticarcinogenic, hepatoprotective, anti-ulcerogenic, anti-allergic, antidiabetic, astringent and anaesthetic (Salomao et al, 2004; Cauich-Kumul & Campos, 2019). As a non-toxic natural product and a valuable source of many biologically active compounds, propolis has been successfully applied as a remedy in traditional and alternative medicine, as a promising food biopreservative (Tosi et al., 2007; Seibert et al., 2019), a nutritional value enhancer (Morsy et al., 2021) and an active ingredient in various cosmetic formulations (Goik et al., 2015; Pasupuleti et al., 2017).

Despite the intensive chemical and pharmacological studies on propolis and the numerous scientific publications in recent years, there is still limited

information concerning Bulgarian propolis. Therefore, the aim of the present study was to investigate and compare the variations in physicochemical parameters, antioxidant and antimicrobial activity of propolis collected from different regions of Bulgaria.

MATERIAL AND METHODS

Raw propolis material

Eighty fresh propolis samples were collected at the end of the active beekeeping season (August-October) from beekeepers located in all twenty-eight districts of Bulgaria in the period of 2020-2022 and then delivered to the laboratory by a courier (Tab. 1). The samples were labelled in order of receipt and then stored in plastic containers at room temperature in darkness until analysis. The samples were analysed in batches shortly after the delivery.

Preparation of propolis extracts

The propolis samples were finely chopped with a blender (Bosch, Germany). In order to prepare 2% propolis extracts, a mass of 0.4 g of each sample was weighed, placed in a plastic tube and macerated with 20 ml of methanol (Sigma-Aldrich, Merck, Germany). Next, the samples were vigorously shaken on vortex V-1 (Biosan, Latvia) for 15-20 s and placed at room temperature for 72 h in darkness. During the extraction, the samples were periodically vortexed. The obtained methanolic extracts were filtered through filter paper and then stored for further analyses (Tumbarski et al., 2021).

Determination of colour

The colour of raw propolis was determined with a portable colorimeter FRU WR-10QC (China) and recorded in L-a-b colour system. The CI-ELAB system consists of a lightness component (L)

Table 1.

Origin of the propolis samples

Sample #	Town/village	District	GPS Coordinates	Climatic zone
1	Stamboliyski	Plovdiv	42°08'N 24°36'E	TrC
2	Silistra	Silistra	44°07'N 27°17'E	TC
3	Simitli	Blagoevgrad	41°54'N 23°08'E	CM
4	Burgas	Burgas	42°30'N 27°28'E	BS
5	Ruse	Ruse	43°50'N 25°57'E	TC
6	Kozarevets	Stara Zagora	42°13'N 25°34'E	TrC
7	Valchedram	Montana	43°45'N 23°26'E	TC
8	Targovishte	Targovishte	43°15'N 26°34'E	TC
9	Matenitsa	Plovdiv	42°19'N 24°22'E	TrC
10	Brezovo	Plovdiv	42°20'N 25°08'E	TrC
11	Krichim	Plovdiv	42°03'N 24°28'E	TrC
12	Gorna Malina	Sofia	42°41'N 23°42'E	TC
13	Shumen	Shumen	43°16'N 26°55'E	TC
14	Varna	Varna	43°13'N 27°55'E	BS
15	Krasnovo	Plovdiv	42°27'N 24°32'E	TrC
16	Asenovgrad	Plovdiv	42°01'N 24°52'E	TrC
17	Batak (Tsarnat)	Pazardzhik	41°57'N 24°13'E	M
18	Batak (St. Troitsa)	Pazardzhik	41°57'N 24°13'E	M
19	Shabla	Dobrich	43°49'N 27°09'E	BS
20	Lenovo	Plovdiv	41°57'N 25°04'E	TrC

21	Hisarya	Plovdiv	42°30’N 24°42’E	TrC
22	Karnobat	Burgas	42°39’N 26°59’E	TrC
23	Dimitrovgrad	Haskovo	42°03’N 25°36’E	TrC
24	Glavan	Stara Zagora	42°03’N 26°07’E	TrC
25	Tsarimir	Plovdiv	42°19’N 24°40’E	TrC
26	Dren	Pernik	42°25’N 23°09’E	TC
27	Negovantsi	Pernik	42°27’N 22°57’E	TC
28	Divlya	Pernik	42°32’N 22°40’E	TrC
29	Vrabcha	Pernik	42°51’N 22°44’E	TC
30	Montana	Montana	43°25’N 23°13’E	TC
31	Bezdenitsa	Montana	43°55’N 23°20’E	TC
32	Barkach	Pleven	43°18’N 24°25’E	TC
33	Pernik	Pernik	42°36’N 23°02’E	TC
34	Osenets	Razgrad	43°33’N 26°22’E	TC
35	Popina	Silistra	44°07’N 26°57’E	TC
36	Loznitsa	Dobrich	43°58’N 27°55’E	TC
37	Debrene	Dobrich	43°23’N 27°51’E	TC
38	Boychinovtsi	Montana	43°27’N 23°22’E	TC
39	Bankya	Sofia	42°42’N 23°08’E	TC
40	Vladimir	Pernik	42°26‘N 23°05‘E	TC
41	Pernik (Tsarkva)	Pernik	42°36’N 23°02’E	TC
42	Petrich	Blagoevgrad	41°24’N 23°13’E	CM
43	Yakoruda	Blagoevgrad	42°01’N 23°41’E	M
44	Pernik (Draganovets)	Pernik	42°36’N 23°02’E	TC
45	Brezovo 2	Plovdiv	42°20’N 25°08’E	TrC
46	Chirpan	Stara Zagora	42°12’N 25°20’E	TrC
47	Razgrad	Razgrad	43°32’N 26°31’E	TC
48	Belovitsa	Plovdiv	42°25’N 24°31’E	TrC
49	Lenovo 2	Plovdiv	41°57’N 25°04’E	TrC
50	Lenovo 3	Plovdiv	41°57’N 25°04’E	TrC
51	Yundola	Pazardzhik	42°03’N 23°52’E	M
52	Dyulevo	Burgas	42°23’N 27°09’E	BS
53	Zagortsi	Burgas	42°24’N 27°04’E	BS
54	Krusheto	V. Tarnovo	43°13’N 25°40’E	TC
55	Dzhurovo	Sofia	42°56’N 24°03’E	M
56	Dolna Dikanya	Pernik	42°25’N 23°07’E	TC
57	Bornarevo	Pernik	42°32’N 22°55’E	TC
58	Cherkovo	Burgas	42°29’N 27°02’E	TrC
59	Nisovo	Ruse	43°39’N 26°04’E	TC
60	Belene	Pleven	43°39’N 25°07’E	TC
61	Aleksandrovo	Shumen	42°59’N 26°58’E	TC
62	Elena	V. Tarnovo	42°56’N 25°53’E	TC
63	Cherven breg	Kyustendil	42°18’N 23°10’E	TrC

64	Gorsko Novo selo	V. Tarnovo	43°05'N 25°56'E	TC
65	Borovitsa	Kardzhali	41°66'N 25°22'E	CM
66	Simeonovgrad	Haskovo	42°02'N 25°50'E	TrC
67	Gamzovo	Vidin	44°05'N 22°45'E	TC
68	Medeshevtsi	Vidin	43°53'N 22°38'E	TC
69	Voynitsa	Vidin	43°56'N 22°41'E	TC
70	Kotel	Sliven	42°53'N 26°27'E	TrC
71	Dolna Kremena	Vratsa	43°10'N 23°44'E	TC
72	Smolyan	Smolyan	41°35'N 24°42'E	CM
73	Bulgarsko Slivovo	V. Tarnovo	43°33'N 25°18'E	TC
74	Parsha	Gabrovo	42°57'N 25°29'E	TC
75	Ritya	Gabrovo	42°59'N 25°25'E	TC
76	Kozi rog	Gabrovo	42°57'N 25°16'E	TC
77	Burya	Gabrovo	43°02'N 25°19'E	TC
78	Yambol	Yambol	42°29'N 26°30'E	TrC
79	Malinovo	Lovech	42°90'N 24°90'E	TC
80	Starosel	Plovdiv	42°29'N 24°34'E	TrC

TC - Temperate continental; TrC - Transitional continental; CM - Continental-Mediterranean; BS - Black Sea; M - Mountain

and two chromatic components, in which the a value represents green (-a) to red (+a) and the b value represents blue (-b) to yellow (+b) colours. The colorimeter was calibrated using a standard white plate (L=96.20, a=0.06, b=-6.20). The values of these parameters were read and recorded automatically by touching the device to the surface of the samples (Falcao et al., 2013). The colour was determined by entering the data from L-a-b in a colour available online (http://www.colormine.org/color-converter). Chroma (C-ab) and hue (h°) values were calculated from the a and b parameters according to Ly et al. (2020).

Determination of pH

The pH was assessed using a pH-meter WTW pH 7110 (InoLab®, Germany). The glass electrode was immersed directly into the propolis extracts at room temperature until a constant value. The calibration was performed with standard buffer solutions (Tumbarski et al., 2021).

Determination of moisture

A moisture balance analyser KERN DAB 100-3 (Kern&Sohn GmbH, Germany) was used to heat a 1 g sample of raw propolis at 110°C to a constant weight in order to determine moisture. The moisture content (%) was automatically read by the analyser based on the difference between the initial weight and the weight after drying of the sample (Pratami et al., 2019).

Test microorganisms

Twenty microorganisms, including eight Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus amyloliquefaciens* 4BCL-YT, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632, *Listeria innocua* ATCC 33090, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Micrococcus luteus* 2YC-YT), five Gram-negative bacteria (*Salmonella enteritidis* ATCC 13076, *Klebsiella* sp. - clinical isolate, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027), two yeasts (*Candida albicans* NBIMCC 74, *Saccharomyces cerevisiae* ATCC 9763) and five fungi *Aspergillus niger* ATCC 1015, *Aspergillus flavus*, *Penicillium* sp., *Rhizopus* sp. - plant isolates, *Fusarium moniliforme* ATCC 38932) from the collection of the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria, were selected for the antimicrobial activity test.

Culture media

Luria-Bertani agar medium with glucose (LBG agar). LBG agar was used for cultivation of test bacteria. A quantity of 50 g of LBG-solid substance mixture (containing 10 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g glucose and 15 g agar) was dissolved in 1 L of deionized water, pH 7.5±0.2.

Malt extract agar (MEA). MEA was used for cultivation of test yeasts and fungi. A quantity of 50 g of the MEA-solid substance mixture (containing 30 g malt extract, 5 g mycological peptone and 15 g agar) was dissolved in 1 L of deionized water, pH 5.4±0.2.

The culture media were prepared according to the manufacturer's instructions (Scharlab SL, Spain) and autoclaved at 121°C for 20 min before use.

Total phenolic content of propolis extracts

The total phenolic content (TPC) was assessed using a Folin-Ciocalteu reagent. The reaction mixture contained 1 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, Merck), 0.8 ml of 7.5% sodium carbonate (Sigma-Aldrich, Merck) and 0.2 ml of the tested propolis extract. The absorbance was measured spectrophotometrically (Camspec M107, Spectronic-Camspec Ltd., UK) at 765 nm after incubation at room temperature for 20 min (in darkness) against a blank (distilled water). The results were presented as mg equivalent of gallic acid (GAE)/g propolis (Ivanov et al., 2014).

Total flavonoid content of propolis extracts

The total flavonoid content (TFC) was determined according to the method described by Ivanov et al. (2014). An aliquot of 1 ml of the propolis extract was added to 0.1 ml of 10% $Al(NO_3)_3$, 0.1 ml of 1 M CH_3COOK and 3.8 ml of distilled water. The absorbance was measured at 415 nm after incubation at room temperature for 40 min using a quercetin as a standard. The results are expressed as mg quercetin equivalents (QE)/g propolis.

Antioxidant activity of propolis extracts

DPPH radical scavenging assay. The reaction mixture containing 2.85 ml of DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) and 0.15 ml of the tested propolis extract was kept at 37°C for 15 min. The reduction of absorbance was measured at 517 nm against a blank (methanol). The antioxidant activity was expressed as mM Trolox® equivalents (TE)/g propolis (Ivanov et al., 2014).

Ferric-reducing antioxidant power (FRAP) assay

The FRAP reagent was freshly prepared with 300 mM acetate buffer with pH 3.6, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid and 20 mM Iron (III) chloride hexahydrate in distilled water in a ratio of 10:1:1. The reaction mixture (3 ml of FRAP reagent and 0.1 ml of the propolis extract) was incubated at 37°C for 10 min in darkness. The absorbance was measured at 593 nm against a blank (distilled water). The antioxidant activity was expressed as mM TE/g propolis (Ivanov et al., 2014).

Antimicrobial activity assay

The antimicrobial activity of propolis extracts was determined by the agar well diffusion method according to Tumbarski et al. (2018). The bacteria *B. subtilis*, *B. amyloliquefaciens* and *M. luteus* were cultured on LBG agar at 30°C for 24 h, while *S. aureus*, *L. monocytogenes*, *L. innocua*, *E. faecalis*, *E. faecium*, *S. enteritidis*, *Klebsiella* sp., *E. coli*, *P. vulgaris* and *P. aeruginosa* were cultured on LBG agar at 37°C for 24 h. The yeast *C. albicans* was cultured on MEA at 37°C, while *S. cerevisiae* was cultured on MEA at 30°C for 24 h. The test fungi *A. niger*, *A. flavus*, *Penicillium* sp., *Rhizopus* sp., and *F. moniliforme* were grown on MEA at 30°C for seven days or until sporulation.

The bacterial/yeast inocula were prepared through homogenization of a small amount of biomass in 5 ml of sterile 0.5% NaCl. The fungal inocula were prepared by the addition of 5 ml of sterile 0.5% NaCl directly into the cultivation tubes. After stirring by vortex V-1 plus (Biosan, Latvia), the fungal inocula were filtered and replaced in other tubes before use. The number of viable cells and fungal spores was determined using a bacterial counting chamber Thoma (Poly-Optik, Germany). Their final concentrations were adjusted to 10^8 cfu/ml for bacterial/yeast cells and 10^5 cfu/ml for fungal spores, and then inoculated in preliminarily melted and tempered at 45-48°C LBG/MEA. Next, the inoculated media

were transferred in a quantity of 18 ml in sterile Petri plates (d=90 mm) (Gosselin™, France) and allowed to harden. Six wells (d=6 mm) per plate were then cut and triplicates of 60 µL of the methanolic propolis extracts were pipetted into the agar wells. The Petri plates were incubated at identical conditions.

The antimicrobial activity was determined through the measuring of the diameter of the inhibition zones (IZ) around the wells on the 24-th and 48-th hour of incubation. Test microorganisms with IZ of 18 mm or more were considered sensitive; moderately sensitive were those in which the IZ were from 12 to 18 mm; resistant were those in which the IZ were up to 12 mm or completely missing.

Statistical analysis

Data from triplicate experiments were processed with MS Office Excel 2010 software using statistical functions to determine the standard deviation (±SD) and maximum estimation error at significance levels $p<0.05$. The correlation (r^2) was calculated with MS Office Excel 2010 software.

RESULTS

Physicochemical parameters - colour, pH and moisture content

The colour of propolis is one of the visible physicochemical characteristics and can be used as an important indicator of its phenolic content and biological activity. The determination of propolis colour was performed through daylight visual observation and colorimetrically. As seen from the results in Tab. 2, the raw propolis samples obtained from all twenty-eight districts of Bulgaria possessed brown or green colour with the corresponding variations in the shade - from light to dark, depending on the climatic zones from which they are collected. The propolis originating from the continental Mediterranean, Black sea and

Table 2.

Colour index of the raw propolis samples

Sample #	Colour	Colour index				
		L	a	b	C-ab	h°
1	Light brown	55.81 ± 5.01	5.02 ± 0.26	8.88 ± 0.11	10.26 ± 2.49	59.45 ± 7.0
2	Light brown	54.44 ± 1.19	5.99 ± 1.02	8.98 ± 1.13	10.80 ± 1.35	56.42 ± 1.8
3	Brown	50.50 ± 5.63	6.80 ± 2.67	6.91 ± 0.63	9.74 ± 4.97	43.24 ± 6.7
4	Dark brown	43.42 ± 2.88	1.54 ± 0.35	1.07 ± 0.35	1.89 ± 0.25	35.40 ± 8.9
5	Dark brown	49.48 ± 0.78	4.72 ± 0.24	3.61 ± 0.16	5.94 ± 0.25	37.46 ± 1.9
6	Dark brown	45.54 ± 1.25	2.75 ± 0.50	3.38 ± 0.91	4.37 ± 0.40	51.17 ± 4.0
7	Dark green	47.93 ± 2.12	2.65 ± 0.87	2.44 ± 0.37	3.61 ± 1.39	41.73 ± 4.1
8	Dark green	47.89 ± 2.30	3.00 ± 1.07	2.58 ± 0.70	4.04 ± 0.74	41.90 ± 4.0
9	Brown	50.23 ± 4.12	4.85 ± 0.54	7.33 ± 0.91	8.82 ± 1.05	56.12 ± 6.7
10	Green	52.68 ± 2.51	1.97 ± 0.47	1.43 ± 0.18	2.44 ± 0.44	36.52 ± 5.3
11	Brown	49.04 ± 1.28	3.67 ± 0.50	3.48 ± 0.47	5.10 ± 0.72	43.08 ± 8.7
12	Light brown	58.22 ± 1.71	6.71 ± 0.73	19.20 ± 3.03	20.35 ± 3.30	70.53 ± 2.8
13	Light brown	54.81 ± 0.84	8.28 ± 0.84	18.86 ± 0.38	20.62 ± 4.63	65.82 ± 3.3
14	Dark brown	42.53 ± 2.15	2.84 ± 0.23	3.67 ± 0.93	4.67 ± 0.74	51.59 ± 7.2
15	Dark green	40.52 ± 0.63	5.01 ± 0.17	8.53 ± 0.55	9.89 ± 0.08	59.58 ± 1.3
16	Dark brown	43.45 ± 2.33	11.07 ± 0.41	6.75 ± 0.19	12.97 ± 0.43	31.35 ± 2.0
17	Brown	50.00 ± 2.27	6.55 ± 1.37	9.13 ± 0.89	11.36 ± 2.65	53.50 ± 1.4
18	Dark brown	38.92 ± 2.22	2.80 ± 0.19	2.74 ± 0.92	3.93 ± 0.22	44.20 ± 5.3
19	Brown	49.74 ± 1.59	5.65 ± 0.30	10.78 ± 1.23	12.21 ± 2.68	61.54 ± 5.9

20	Dark brown	46.75 ± 0.66	7.53 ± 0.73	13.52 ± 1.74	15.48 ± 0.83	60.92 ± 1.4
21	Brown	51.85 ± 0.51	6.19 ± 0.14	10.67 ± 0.42	12.34 ± 0.10	59.86 ± 1.0
22	Green	51.94 ± 0.13	5.34 ± 0.48	12.78 ± 2.52	13.85 ± 1.00	67.34 ± 0.4
23	Dark brown	42.41 ± 2.58	3.98 ± 0.33	5.10 ± 0.72	6.49 ± 0.72	51.71 ± 5.7
24	Dark brown	39.84 ± 2.33	4.47 ± 0.42	3.70 ± 0.97	5.89 ± 0.41	39.06 ± 2.3
25	Light brown	52.49 ± 0.12	6.80 ± 0.36	11.38 ± 0.56	13.27 ± 1.65	58.92 ± 2.9
26	Dark brown	47.51 ± 1.03	5.98 ± 0.54	6.12 ± 1.91	8.57 ± 0.18	45.66 ± 4.4
27	Light brown	54.67 ± 0.65	7.85 ± 0.58	19.58 ± 1.00	21.10 ± 2.56	68.07 ± 1.0
28	Light brown	53.74 ± 0.42	7.03 ± 0.67	26.43 ± 1.21	27.36 ± 0.63	75.09 ± 1.6
29	Brown	50.11 ± 3.31	3.55 ± 0.14	3.08 ± 0.51	4.73 ± 0.70	40.18 ± 8.1
30	Light brown	54.82 ± 2.07	4.41 ± 0.12	7.62 ± 0.78	8.80 ± 0.55	59.86 ± 1.1
31	Dark brown	45.66 ± 4.31	6.31 ± 1.78	7.36 ± 0.65	9.71 ± 3.32	48.42 ± 4.8
32	Dark brown	45.90 ± 1.14	4.64 ± 0.21	7.92 ± 1.18	9.18 ± 0.96	59.49 ± 2.3
33	Brown	50.86 ± 4.13	6.60 ± 0.84	19.00 ± 1.62	20.12 ± 1.39	70.88 ± 1.4
34	Dark brown	36.65 ± 3.55	6.36 ± 0.15	11.61 ± 2.16	13.25 ± 0.38	61.25 ± 1.6
35	Dark brown	44.85 ± 1.45	6.69 ± 0.22	11.45 ± 0.41	13.27 ± 0.79	59.66 ± 0.9
36	Light brown	54.01 ± 0.77	4.71 ± 1.11	8.46 ± 0.88	9.70 ± 1.07	61.19 ± 4.3
37	Dark brown	45.05 ± 2.35	5.11 ± 0.36	8.82 ± 0.20	10.19 ± 1.19	59.78 ± 1.9
38	Brown	51.89 ± 1.99	3.86 ± 0.72	6.47 ± 1.23	7.53 ± 1.75	58.95 ± 1.9
39	Light brown	55.57 ± 1.02	5.55 ± 0.62	16.78 ± 1.52	17.68 ± 2.16	71.61 ± 2.1
40	Brown	50.91 ± 2.03	6.47 ± 0.92	19.18 ± 0.39	20.25 ± 0.66	71.40 ± 2.2
41	Dark brown	42.34 ± 1.14	3.92 ± 0.33	10.73 ± 0.23	11.42 ± 0.85	69.88 ± 2.0
42	Dark brown	45.48 ± 2.68	1.99 ± 0.77	9.29 ± 0.48	2.01 ± 0.78	8.17 ± 0.5
43	Dark brown	45.96 ± 1.63	8.17 ± 0.41	16.23 ± 0.59	18.17 ± 1.27	63.24 ± 0.8
44	Dark brown	48.31 ± 0.97	8.54 ± 3.49	17.69 ± 0.43	19.77 ± 2.66	64.78 ± 7.7
45	Dark brown	45.51 ± 5.21	8.26 ± 0.38	19.84 ± 0.34	21.49 ± 0.34	67.39 ± 1.1
46	Brown	51.65 ± 2.20	7.47 ± 0.21	17.97 ± 0.43	19.46 ± 0.22	67.42 ± 0.6
47	Dark brown	36.37 ± 0.70	4.89 ± 0.33	9.04 ± 0.81	10.28 ± 0.56	61.58 ± 0.8
48	Light brown	59.75 ± 4.77	8.95 ± 0.78	25.06 ± 0.48	26.62 ± 0.81	70.37 ± 1.2
49	Green	49.88 ± 0.37	5.23 ± 0.61	18.19 ± 0.11	18.93 ± 0.41	73.97 ± 1.9
50	Light brown	57.09 ± 0.45	7.38 ± 0.20	21.91 ± 1.13	23.12 ± 0.37	71.39 ± 0.3
51	Dark brown	32.27 ± 1.19	4.61 ± 0.28	7.45 ± 0.63	8.76 ± 0.47	58.22 ± 1.4
52	Brown	51.08 ± 0.87	7.75 ± 0.24	14.80 ± 1.35	16.71 ± 0.82	62.36 ± 0.6
53	Light brown	52.27 ± 0.20	8.86 ± 0.22	23.28 ± 0.16	24.91 ± 0.39	69.15 ± 0.8
54	Brown	50.37 ± 3.19	5.66 ± 1.37	17.21 ± 0.55	18.14 ± 0.70	71.85 ± 4.0
55	Light brown	51.51 ± 2.63	5.24 ± 1.09	8.73 ± 2.39	10.19 ± 2.58	58.79 ± 2.7
56	Light brown	53.77 ± 2.43	5.86 ± 2.16	20.22 ± 3.94	21.07 ± 4.38	74.16 ± 2.7
57	Brown	48.19 ± 0.55	7.99 ± 2.12	17.57 ± 1.81	19.33 ± 2.49	65.84 ± 3.8
58	Brown	52.23 ± 2.31	5.85 ± 2.15	22.27 ± 4.19	23.04 ± 4.56	75.62 ± 2.9
59	Dark brown	45.15 ± 6.27	2.10 ± 0.60	1.94 ± 0.31	2.86 ± 0.64	43.27 ± 4.0
60	Green	50.94 ± 3.92	4.71 ± 0.74	9.21 ± 1.59	10.35 ± 1.71	62.83 ± 2.2
61	Green	53.08 ± 6.74	4.96 ± 1.89	14.55 ± 2.79	15.46 ± 5.76	69.90 ± 8.3
62	Dark brown	39.47 ± 0.80	2.48 ± 0.25	3.41 ± 0.49	4.23 ± 0.33	53.76 ± 5.9

63	Light brown	51.50 ± 3.72	11.30 ± 2.73	25.99 ± 1.96	28.42 ± 1.93	66.55 ± 5.7
64	Light brown	51.87 ± 3.44	7.94 ± 1.45	20.84 ± 1.67	22.31 ± 2.05	69.26 ± 2.1
65	Light brown	56.38 ± 0.64	5.30 ± 1.71	21.35 ± 4.66	22.01 ± 4.93	76.24 ± 1.4
66	Light brown	59.04 ± 1.62	5.99 ± 0.61	19.20 ± 2.67	20.12 ± 2.72	72.63 ± 0.8
67	Green	51.60 ± 0.90	5.88 ± 0.72	14.02 ± 2.76	15.25 ± 2.42	66.73 ± 5.5
68	Green	53.48 ± 1.49	6.17 ± 0.53	21.50 ± 0.39	22.37 ± 0.40	74.00 ± 1.3
69	Green	52.38 ± 1.32	7.04 ± 0.22	22.33 ± 1.39	23.42 ± 1.39	72.48 ± 0.6
70	Dark brown	40.23 ± 0.45	4.66 ± 0.17	9.67 ± 0.24	10.73 ± 0.27	64.27 ± 0.5
71	Brown	47.02 ± 1.20	9.45 ± 0.34	16.31 ± 0.24	18.85 ± 0.37	59.92 ± 0.6
72	Brown	45.62 ± 1.21	6.81 ± 0.80	10.21 ± 0.97	12.27 ± 1.16	56.33 ± 2.3
73	Dark green	35.00 ± 4.78	4.46 ± 0.84	12.76 ± 2.18	13.52 ± 2.33	70.77 ± 0.6
74	Green	48.95 ± 2.70	5.11 ± 0.38	18.45 ± 1.60	19.15 ± 1.62	74.50 ± 0.8
75	Green	46.15 ± 0.95	6.76 ± 0.51	16.41 ± 0.21	17.75 ± 0.37	67.63 ± 1.3
76	Dark brown	41.99 ± 5.17	7.49 ± 1.08	16.02 ± 2.23	17.72 ± 5.15	63.96 ± 5.1
77	Green	55.15 ± 0.25	6.71 ± 2.49	11.58 ± 1.64	13.43 ± 2.67	60.64 ± 5.5
78	Dark green	43.56 ± 3.52	5.55 ± 1.26	9.43 ± 1.06	10.95 ± 1.54	59.80 ± 3.1
79	Green	51.85 ± 1.86	4.09 ± 0.46	6.27 ± 0.38	7.49 ± 0.57	56.93 ± 1.4
80	Brown	45.66 ± 1.00	4.39 ± 0.33	11.79 ± 0.95	12.59 ± 0.83	69.47 ± 2.6

Table 3.

pH and moisture content the propolis samples

Parameter	Minimum	Maximum	Average*
pH	4.82 ± 0.02	5.87 ± 0.01	5.32 ± 0.18
Moisture, %	0.98 ± 0.25	2.97 ± 0.35	1.76 ± 0.41

* - mean value (n = 80)

mountain climatic zones showed brown colour, while the propolis originating from the temperate continental and transitional continental climatic zones exhibited brown or green colour. The ratio of the two propolis colours was as follows: temperate continental zone - 29.3% green and 70.7% brown; transitional continental zone - 20% green and 80% brown.

The pH and moisture content of propolis are physicochemical parameters highly dependent on the relative air humidity, temperature and plant diversity of the certain geographical area. The results presented in Tab. 3 demonstrate that the pH and moisture content of the tested propolis samples varied within narrow limits, regardless of their origin. pH values varied from 4.82 (sample 33) to 5.87 (sample 67). The moisture content ranged from 0.98% (sample 1) to 2.97% (sample 70), which was in agreement with the data in the literature.

Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity

Propolis contains a great variety of chemical compounds, mainly polyphenols (flavonoids, phenolic acids and their esters), which contribute to its biological activity, in particular its antioxidant potential. As seen from the results in Tab. 4, the TPC of the different propolis samples showed great variations - from 63.14 mg GAE/g (sample 41) to 737.27 mg GAE/g (sample 77).

Table 4.

TPC, TFC and antioxidant activity of the methanolic propolis extracts (20 mg/ml)

Sample #	Polyphenols, mg GAE/g	Flavonoids, mg QE/g	Antioxidant activity	
			DPPH, mM TE/g	FRAP, mM TE/g
1	172.89 ± 0.55	90.10 ± 0.39	982.01 ± 4.98	584.33 ± 1.62
2	212.77 ± 0.80	129.24 ± 4.99	1080.27 ± 5.48	738.66 ± 1.78
3	536.33 ± 1.00	182.59 ± 4.02	1598.66 ± 3.67	966.77 ± 2.44
4	72.30 ± 0.53	59.07 ± 0.38	18.56 ± 1.70	136.26 ± 1.26
5	111.56 ± 0.38	63.95 ± 1.63	372.21 ± 5.27	368.55 ± 2.58
6	183.66 ± 0.36	113.53 ± 1.13	1064.84 ± 7.28	637.44 ± 1.32
7	164.98 ± 0.60	90.58 ± 1.20	746.78 ± 2.72	566.27 ± 2.08
8	121.77 ± 1.36	73.64 ± 0.18	761.13 ± 4.62	440.20 ± 3.62
9	159.15 ± 0.41	85.24 ± 1.82	692.42 ± 5.60	548.80 ± 2.88
10	160.30 ± 0.75	83.36 ± 1.53	885.39 ± 5.09	535.41 ± 3.27
11	110.83 ± 0.91	121.17 ± 0.57	514.78 ± 3.89	954.01 ± 6.28
12	216.91 ± 0.62	132.88 ± 4.77	998.45 ± 3.03	1208.81 ± 5.25
13	191.39 ± 0.55	107.92 ± 0.39	946.54 ± 5.17	1188.24 ± 5.33
14	133.79 ± 0.54	73.94 ± 1.53	534.07 ± 2.47	715.80 ± 4.47
15	129.46 ± 0.53	71.18 ± 1.34	662.57 ± 5.38	839.27 ± 2.42
16	181.81 ± 0.56	90.84 ± 1.39	949.44 ± 4.88	1067.59 ± 1.04
17	136.24 ± 0.98	68.06 ± 1.36	575.78 ± 5.31	792.54 ± 2.27
18	102.14 ± 0.86	49.24 ± 1.81	512.78 ± 6.34	637.39 ± 4.58
19	167.08 ± 0.94	82.11 ± 1.79	816.89 ± 3.04	1046.72 ± 1.66
20	127.53 ± 0.34	69.59 ± 1.29	624.89 ± 6.00	773.27 ± 2.99
21	161.80 ± 0.79	92.06 ± 1.95	769.91 ± 4.32	1008.77 ± 5.01
22	160.23 ± 0.79	74.42 ± 1.00	616.31 ± 6.77	889.43 ± 5.78
23	117.84 ± 1.02	60.48 ± 1.08	572.03 ± 4.56	507.99 ± 1.81
24	133.63 ± 0.41	76.41 ± 0.44	587.83 ± 4.27	564.24 ± 7.34
25	134.74 ± 0.21	77.44 ± 0.88	568.52 ± 7.02	580.02 ± 4.83
26	161.10 ± 1.23	92.70 ± 1.76	725.14 ± 2.30	726.39 ± 3.79
27	110.50 ± 0.81	48.08 ± 0.21	341.12 ± 1.62	439.23 ± 5.39
28	157.02 ± 0.62	94.37 ± 2.40	569.24 ± 3.38	629.84 ± 4.53
29	144.70 ± 0.62	74.17 ± 1.52	521.70 ± 3.02	616.84 ± 7.29
30	143.96 ± 0.80	68.23 ± 1.90	477.54 ± 3.48	574.52 ± 2.47
31	151.05 ± 1.05	83.11 ± 0.88	600.98 ± 7.05	689.24 ± 2.48
32	137.32 ± 0.82	65.73 ± 0.43	479.85 ± 6.94	600.13 ± 2.11
33	158.38 ± 0.42	70.96 ± 1.99	580.17 ± 3.21	647.47 ± 2.02
34	146.42 ± 0.21	76.27 ± 2.47	608.38 ± 4.32	616.81 ± 4.45
35	137.84 ± 0.42	72.93 ± 2.55	543.90 ± 2.98	639.19 ± 4.04
36	141.08 ± 0.87	54.90 ± 0.69	531.72 ± 4.91	636.80 ± 4.61
37	145.39 ± 1.24	65.93 ± 2.84	451.22 ± 3.19	590.35 ± 2.61

38	175.85 ± 0.62	123.80 ± 3.50	880.94 ± 6.44	925.23 ± 3.61
39	179.40 ± 0.38	86.82 ± 0.61	749.55 ± 2.52	835.86 ± 5.34
40	213.16 ± 0.34	97.85 ± 2.54	816.25 ± 5.11	811.95 ± 2.24
41	63.14 ± 0.85	29.22 ± 1.80	21.25 ± 1.16	98.91 ± 0.57
42	93.69 ± 0.51	60.94 ± 1.26	76.79 ± 1.15	82.28 ± 1.69
43	133.38 ± 1.03	57.93 ± 0.18	680.20 ± 2.27	404.47 ± 4.59
44	145.05 ± 0.57	84.30 ± 3.01	606.80 ± 3.37	514.01 ± 4.89
45	157.82 ± 0.66	91.60 ± 2.09	623.41 ± 4.92	551.75 ± 5.86
46	160.13 ± 0.56	99.31 ± 3.14	673.64 ± 2.52	510.15 ± 6.52
47	135.15 ± 0.88	77.85 ± 0.56	586.01 ± 5.89	470.76 ± 4.08
48	119.58 ± 0.73	57.16 ± 0.38	450.65 ± 2.39	407.17 ± 1.15
49	165.38 ± 0.27	104.41 ± 1.28	701.95 ± 4.97	536.42 ± 4.80
50	120.14 ± 0.61	54.92 ± 1.34	319.64 ± 2.27	357.21 ± 1.79
51	152.67 ± 0.60	78.13 ± 0.84	543.50 ± 4.36	510.88 ± 3.54
52	177.78 ± 1.85	124.61 ± 0.95	1013.64 ± 3.07	1009.48 ± 2.00
53	158.35 ± 1.78	94.02 ± 0.48	898.27 ± 2.98	981.19 ± 3.01
54	154.72 ± 2.10	79.96 ± 0.88	707.57 ± 5.29	644.32 ± 2.19
55	125.49 ± 0.47	72.20 ± 0.99	691.62 ± 2.09	719.70 ± 2.03
56	135.91 ± 0.44	68.33 ± 1.15	833.28 ± 3.60	513.52 ± 2.38
57	162.14 ± 0.80	84.99 ± 1.45	958.49 ± 2.46	639.48 ± 5.09
58	148.78 ± 1.76	94.15 ± 1.39	982.84 ± 1.94	643.46 ± 2.50
59	148.04 ± 0.11	90.10 ± 0.47	1145.43 ± 6.85	695.37 ± 3.62
60	125.72 ± 2.84	62.30 ± 1.61	902.38 ± 4.28	768.80 ± 4.55
61	89.25 ± 0.96	54.22 ± 1.13	360.69 ± 1.92	574.67 ± 1.38
62	127.01 ± 3.33	74.98 ± 1.58	694.29 ± 6.99	376.74 ± 2.77
63	190.83 ± 3.46	152.69 ± 1.18	1026.52 ± 2.15	594.17 ± 3.62
64	131.54 ± 0.70	64.26 ± 0.50	668.90 ± 2.82	552.82 ± 2.66
65	154.95 ± 3.57	89.97 ± 1.10	440.13 ± 1.09	460.33 ± 2.96
66	194.26 ± 3.67	98.48 ± 0.72	538.23 ± 5.63	538.87 ± 4.15
67	197.35 ± 1.50	102.36 ± 0.49	955.39 ± 2.99	758.80 ± 6.43
68	170.02 ± 0.70	85.07 ± 0.74	891.30 ± 1.56	763.06 ± 5.26
69	145.10 ± 0.11	76.52 ± 0.67	534.79 ± 1.57	482.80 ± 2.55
70	124.69 ± 0.34	78.59 ± 0.47	672.02 ± 2.77	461.11 ± 3.75
71	159.38 ± 1.60	93.35 ± 1.44	753.68 ± 4.20	531.95 ± 4.60
72	152.72 ± 0.96	84.53 ± 0.90	517.65 ± 3.89	640.56 ± 1.00
73	163.78 ± 1.43	96.37 ± 0.69	1165.56 ± 3.74	918.94 ± 7.80
74	188.50 ± 1.75	100.04 ± 0.65	1192.44 ± 7.60	863.00 ± 2.63
75	234.18 ± 3.30	129.85 ± 0.70	902.11 ± 1.94	842.92 ± 2.33
76	234.29 ± 1.46	151.53 ± 1.42	1459.09 ± 3.63	1012.26 ± 3.61
77	737.27 ± 1.08	234.17 ± 1.14	1464.75 ± 3.46	916.64 ± 5.66
78	103.20 ± 2.32	50.12 ± 1.40	397.38 ± 2.01	522.24 ± 5.90
79	206.25 ± 0.86	110.31 ± 0.46	1149.19 ± 2.45	884.08 ± 3.32
80	158.50 ± 3.71	83.74 ± 0.85	832.65 ± 4.99	959.66 ± 4.88

The highest TPC values were determined in samples 2, 3, 6, 12, 13, 16, 40, 63, 66, 67, 74, 75, 76, 77 and 79. The TFC also varied in wide limits - between 29.22 mg QE/g (sample 41) and 234.17 mg QE/g (sample 77). The highest TFC values were determined in samples 2, 3, 6, 11, 12, 13, 38, 49, 52, 63, 67, 74, 75, 76, 77 and 79. Consequently, in the most propolis samples, the highest TPC values corresponded to the highest TFC values. The results presented in Tab. 4 demonstrated that the antioxidant activity of propolis samples determined by the DPPH method varied from 18.56 mM TE/g (sample 4) to 1598.66 mM TE/g (sample 3). The highest antiradical activity was exhibited by the samples 1, 2, 3, 6, 12, 13, 16, 52, 57, 58, 59, 60, 63, 67, 73, 74, 75, 76, 77 and 79. The results obtained by the second method - FRAP, showed that the values varied from 82.28 mM TE/g (sample 42) to 1208.81 mM TE/g (sample 12). The highest reducing power possessed samples 3, 11, 12, 13, 16, 19, 21, 38, 52, 53, 73, 74, 76, 77 and 80. In most of the propolis samples, the highest antioxidant values coincided with the highest phenolics and flavonoids concentrations.

Correlation between the TPC, TFC and the antioxidant activity

The antioxidant capacity is a property dependent, to the greatest extent, on the TPC and TFC. In statistical analysis, the correlation indicates the degree to which a pair of variables are linearly related. In this regard, the correlation shows the relationship between the antioxidant activities determined by the different methods, as well as the relationship between them and the TPC and TFC of the propolis samples. The correlation between the TPC, TFC and antioxidant activity of propolis samples measured by the two independent methods (DPPH and FRAP) is presented in Tab. 5. A positive linear correlation (r^2) between the results obtained by the different methods was observed. The highest correlation coefficient (0.85) was determined between the TPC/TFC of the propolis samples. The interrelationship between the colour, colour components (L, a, b, C-ab, and h°), TPC, TFC and the antioxidant activity of the propolis samples is shown in Tab. 6.

The studied propolis samples were divided into two main types in terms of their visible colour: brown (light brown, brown and dark brown) and green (green and dark green). The statistical analysis of the results showed that propolis possessing the colour green had higher TPC and TFC, and antioxidant activity (determined by DPPH assay), compared to propolis with the colour brown. In contrast, the propolis with the colour brown exhibited the highest antioxidant activity determined by FRAP assay. From these five groups, statistically ($p<0.05$) the lowest results for TPC and TFC demonstrated propolis with dark green and dark brown colour (Tab. 6).

The colour characteristics of the investigated propolis samples were an indicator of their TPC, TFC and antioxidant potential. The colour component L in the studied propolis samples ranged between 30 and 60. From the obtained results, it can be seen that as L increased, the mean values of TPC, TFC and antioxidant activity also increased. Statistically significant

Table 5.

Correlation between the TPC, TFC and antioxidant activity of the propolis samples

Methods	Correlation, r^2
TPC / TFC	0.85
TPC / DPPH	0.68
TPC / FRAP	0.44
TFC / DPPH	0.78
TFC / FRAP	0.55
DPPH / FRAP	0.68

Table. 6.

Interrelationship between the colour, colour components (L, a, b, C-ab, and h°), TPC, TFC, and antioxidant activity of the propolis samples

Parameter	Groups				
Colour	**LB**	**B**	**DB**	**G**	**DG**
TPC	157.46 ± 32.57	179.87 ± 91.20	136.86* ± 34.40	214.96 ± 168.8	136.64* ± 27.07
TFC	87.85 ± 27.88	96.81 ± 32.30	76.50 ± 22.68	101.42 ± 46.93	76.38 ± 18.20
DPPH	698.2 ± 239.0	789.0 ± 304.6	605.9 ± 331.1	879.4 ± 302.7	746.4 ± 276.0
FRAP	665.4 ± 235.5	771.9 ± 190.7	555.6 ± 236.4	734.6 ± 159.2	657.2 ± 209.4
L	≤40	40-45	45-50	50-55	≥55
TPC	137.26 ± 19.92	138.34 ± 50.35	150.16 ± 28.20	169.07* ± 27.78	236.93 ± 204.0
TFC	75.61 ± 14.84	76.17 ± 31.51	85.81 ± 19.02	89.78 ± 29.13	105.56 ± 54.48
DPPH	671.1 ± 202.8	638.3 ± 438.8	679.7 ± 236.5	773.0 ± 258.1	742.6 ± 370.7
FRAP	585.0 ± 150.3	623.9 ± 319.7	626.6 ± 215.0	716.4 ± 185.5	663.5 ± 331.4
a	≤2	2-4	4-6	6-8	≥8
TPC	110.32 ± 48.53	136.63 ± 33.22	154.49 ± 23.05	199.14* ± 85.05	159.73* ± 24.95
TFC	67.79 ± 13.52	81.24 ± 28.56	81.51 ± 17.65	98.16* ± 42.83	92.20 ± 28.23
DPPH	327.0 ± 484.1	664.1 ± 295.5	714.8 ± 239.0	774.7** ± 338.2	770.2 ± 194.9
FRAP	251.0 ± 247.4	597.6 ± 231.8	665.2 ± 169.7	730.3** ± 211.1	693.2 ± 300.2
b	≤5	5-10	10-15	15-20	≥20
TPC	133.38 ± 28.87	166.48 ± 85.71	183.22 ± 151.20	174.15* ± 34.50	148.38 ± 21.36
TFC	78.90 ± 19.97	82.78 ± 35.11	86.14 ± 35.95	96.25* ± 26.76	84.68 ± 27.05
DPPH	637.9 ± 308.0	706.0 ± 360.0	709.4 ± 371.1	795.1** ± 274.3	692.0 ± 244.9
FRAP	553.5 ± 195.6	655.7 ± 206.0	653.9 ± 206.9	709.4** ± 253.4	580.3 ± 175.9
C-ab	≤5	5-10	10-15	15-20	≥20
TPC	132.46 ± 33.64	175.89 ± 102.6	173.94 ± 100.62	166.36* ± 35.43	157.83* ± 28.90
TFC	76.69 ± 17.64	89.20 ± 33.48	85.24 ± 32.15	95.46* ± 22.75	87.74 ± 26.40
DPPH	632.9 ± 356.1	692.5 ± 313.2	725.4 ± 272.7	827.6** ± 216.9	691.9 ± 234.9
FRAP	494.3 ± 216.1	686.3 ± 187.4	721.0 ± 235.0	686.6** ± 175.2	653.8 ± 249.4
h°,	≤40	40-50	50-60	60-70	≥70
TPC	128.12 ± 42.64	182.31 ± 134.3	153.91 ± 28.86	175.57 ± 115.49	157.86 ± 29.96
TFC	72.06 ± 12.98	95.50 ± 37.82	84.09 ± 21.36	90.73 ± 40.12	85.69 ± 19.94
DPPH	470.7 ± 392.9	799.1 ± 356.1	704.2 ± 223.1	754.5 ± 298.8	712.7 ± 262.3
FRAP	467.3 ± 361.8	693.1 ± 174.7	645.6 ± 143.9	708.4 ± 250.8	657.4 ± 211.7

LB - light brown; B - brown; DB - dark brown; G - green; DG - dark green; * Statistically significant difference at $p<0.05$ (within the group);

** Statistically significant difference at $p<0.01$ (within the group).

differences ($p<0.05$) were reported at L>50. The red colour component a was also indicative for the amount of TPC and TFC. As the values of a increased, the amount of TPC also increased, and statistically, the propolis samples with a between 6 and 8 had the highest average amounts. The yellow colour component b showed a similar influence to a. The maximum TPC, TFC and antioxidant activity in propolis with a yellow colour component b were observed in samples

Table 7.

Antimicrobial activity of the methanolic propolis extracts (20 mg/ml)

Test microorganism	Inhibition zones (IZ), mm*			
	Minimum	Maximum	Average	MeOH (control)
B. subtilis ATCC 6633	15	25	18.91 ± 1.63	0
B. amyloliquefaciens 4BCL-YT	8	19	15.43 ± 1.56	0
S. aureus ATCC 25923	13	25	19.95 ± 3.05	0
L. monocytogenes NBIMCC 8632	12	30	19.65 ± 3.51	0
L. innocua ATCC 33090	12	24	18.15 ± 2.71	0
E. faecalis ATCC 19433	12	19	14.74 ± 1.83	0
E. faecium ATCC 19434	11	18	14.88 ± 1.35	0
M. luteus 2YC-YT	15	28	19.99 ± 2.56	0
S. enteritidis ATCC 13076	11	18	14.09 ± 1.15	0
Klebsiella sp. (clinical isolate)	8	11	8.94 ± 0.94	0
E. coli ATCC 25922	12	20	16.12 ± 1.71	0
P. vulgaris ATCC 6380	8	12	8.56 ± 1.06	0
P. aeruginosa ATCC 9027	8	22	13.41 ± 4.96	0
C. albicans NBIMCC 74	8	15	12.10 ± 1.37	0
S. cerevisiae ATCC 9763	9	17	12.85 ± 1.53	0
A. niger ATCC 1015	10	16	12.56 ± 1.11	0
A. flavus (plant isolate)	10	20	14.30 ± 2.73	0
Penicillium sp. (plant isolate)	9	18	13.11 ± 1.32	0
Rhizopus sp. (plant isolate)	10	30	17.72 ± 5.68	0
F. moniliforme ATCC 38932	8	17	12.27 ± 2.17	0

* - d_{well} = 6 mm

with values between 15 and 20 ($p<0.05$ for TPC and TFC, and $p<0.1$ for DPPH and FRAP). The results for C-ab and h° were similar. The greatest amounts of TPC, TFC and antioxidants were observed in propolis samples with values of C-ab between 15 and 20, and h° between 60 and 70, respectively, compared to propolis possessing different characteristics (Tab. 6).

Antimicrobial activity

As seen from the results presented in Tab. 7, the methanolic propolis extracts in concentration of 20 mg/ml demonstrated the highest antimicrobial activity against Gram-positive bacteria. The inhibitory effect was most pronounced on *M. luteus* 2YC-YT, *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923. The tested propolis extracts also exhibited significant antilisterial effect on *L. monocytogenes* NBIMCC 8632 and *L. innocua* ATCC 33090. The antimicrobial activity against *B. amyloliquefaciens* 4BCL-YT, *E. faecalis* ATCC 19433 and *E. faecuim* ATCC 19434 was moderate.

Regarding the Gram-negative bacteria, the propolis extracts exhibited moderate antimicrobial activity against *S. enteritidis* ATCC 13076, *E. coli* ATCC 25922 (except samples 1-22 - without an inhibitory effect) and *P. aeruginosa* ATCC 9027 (except samples 6, 8, 23-31, 33-36, 43, 44, 46, 48, 50 and 53, which had no inhibitory activity). The antimicrobial effect on *P. vulgaris* ATCC 6380 and *Klebsiella* sp. was weak. The samples 6-11, 24, 27, 31, 33, 34, 48-54 and 58-67 had no inhibitory effect on *P. vulgaris* ATCC 6380, while the samples 11, 30, 31, 33, 36, 37, 42, 43, 50, 51, 53-60, 63-70, 72, 73, 76, 78

and 79 did not inhibit *Klebsiella* sp.

The propolis extracts demonstrated the highest antifungal activity against *Rhizopus* sp. (except samples 35-53, which did not inhibit the fungal growth) and *A. flavus* (except samples 4-6 and 42 - without an inhibitory effect). A moderate inhibitory effect was observed on the fungi *Penicillium* sp. (except samples 62 and 64 without an inhibitory effect), *A. niger* ATCC 1015, *F. moniliforme* ATCC 38932, and yeasts *S. cerevisiae* ATCC 9763 and *C. albicans* NBIMCC 74. Methanol used as a solvent did not show an antimicrobial effect on the used test microorganisms.

DISCUSSION

As mentioned above, the colour of propolis exhibits a broad range of varieties depending on the vegetable source and geographical location. Brown and dark brown colours were reported for propolis originating from two regions in Morocco (Aboulghazi et al., 2022). Dias et al. (2012) examined twelve propolis samples from four locations of Portugal and found that all samples were heterogeneic in their consistency and colour. Propolis from the Mirandela region showed the colour green, from Mogadouro region - the colour red-yellow, from Nogueira region - the colour red-brown and from Vinhais region - the colour brown.

Findings similar to our results were obtained for propolis from such countries of the Mediterranean region as Palestina and Morocco (pH 4.2-5.2 and moisture 1.01%-2.79%) (Touzani et al., 2021), the northeast part of Portugal (pH 4.7-5.3) (Dias et al., 2012) and Egypt (moisture 2.46%) (Hemeida & Kobeasy, 2003). The results (pH 4.8-5.9 and moisture 1.09-2%) obtained for Moroccan propolis by Aboulghazi et al. (2022) were also in agreement with our results. However, the obtained pH values for Bulgarian propolis were higher in comparison with other data reported for propolis from Poland and Lithuania (Adaškeviciute et al., 2019). The moisture content of our samples was lower than those of propolis collected from the northern parts of India (4.89-7.37%) (Pant et al., 2021).

In recent years, numerous studies have been performed on the TPC, TFC and antioxidant capacity of propolis. Our results were in agreement with the previously published data regarding Bulgarian propolis. Kumazawa et al. (2004) ascertained that TPC and TFC values of the Bulgarian sample were 220 mg GAE/g and 157 mg QE/g, respectively. The study by Zarate et al. (2018) on propolis originating from various areas of Guanajuato state, Mexico, revealed that the TPC ranged from 68 to 500 mg of caffeic acid equivalents (CAE)/g of propolis, while the TFC varied from 13 to 379 mg QE/g of propolis. The antioxidant activity of the same propolis samples also varied and showed values from 39.8 to 54.4 mM TE/g for the DPPH assay, which are lower than our results, and from 50 to 2000 mM TE/g for the FRAP assay, which is comparable to our findings. The amounts of TPC (48.5 to 238.9 mg GAE/g) obtained by Wang et al. (2016) for twenty propolis samples from different regions of South Korea were similar to our results, but the TFC in the same propolis samples showed lower values (20.8-49.8 mg QE/g) that differed significantly from our data. However, the authors stated that their results were in correspondence with those obtained for Australian, Brazilian and Chinese propolis. Similar results to ours were obtained for propolis from Palestina and Morocco (TPC 74.71-148 mg GAE/g and TFC 26.97-118 mg QE/g) (Touzani et al., 2021). Özkök et al. (2021) examined propolis from twenty-three regions of Turkey and determined that the TFC in the tested samples was between 21.28 and 152.56 mg catechin equivalent (CE)/g, while TPC was found between 34.53 and 259.4 mg GAE/g of propolis. In contrast, Socha et al. (2014) analysed the phenolic and flavonoid contents, as well as the antioxidant properties of nine propolis samples from different regions of Poland and reported that the TPC of propolis samples ranged from 150.05 to 197.14 mg/g GAE, while TFC varied from 35.64 to 62.04 mg QE/g, whose values were lower compared to our propolis samples. The propolis samples from Poland also exhibited

lower antiradical activity measured by the DPPH method (1.92-2.69 mM TE/g) and lower reducing power determined by the FRAP method (6.23-9.19 mM Fe(II)/g) in comparison with the Bulgarian propolis samples.

Asem et al. (2019) detected a strong positive correlation between the TPC, TFC and antioxidant activities of Malaysian propolis samples, ranging from 0.763 to 0.971. The same correlation trend between the TPC and TFC (0.882), as well as coefficient values of 0.943 and 0.926 between the FRAP assay and the TPC and TFC were observed. The DPPH assay also demonstrated a positive correlation of TPC and TFC with coefficient values of 0.842 and 0.820, respectively. A positive correlation (r^2) between the TPC and DPPH values (0.8387), and the TPC and FRAP values (0.3522) was determined by Mihai et al. (2011) who examined twenty propolis samples from Transylvania, Romania.

The results from the antimicrobial activity of propolis extracts were in agreement with some previously published studies on this topic. Popova et al. (2011) determined high antimicrobial activity against the pathogens *S. aureus* (IZ 15-27 mm) and moderate activity against *C. albicans* (IZ 12-18 mm) of seventeen propolis samples originating from Malta and one sample from Bulgaria. Comparable to our results were also those obtained by Velikova et al. (2000), who examined the chemical composition and properties of propolis from the Mediterranean region (Bulgaria, Greece, Turkey and Algeria). The authors determined the highest antimicrobial activity against *S. aureus* (IZ 18.7-21.7 mm), and moderate activity against *C. albicans* (IZ 12-17 mm) and *E. coli* (IZ 12-14 mm). In contrast, Afrouzan et al. (2018) analysed propolis from four regions of Iran and established that ethanolic extracts demonstrated antimicrobial effect on *E. coli* ATTC 25922 (IZ 8.33-10 mm), *S. aureus* ATCC 6538 (IZ 8.67-10 mm) and *C. albicans* ATCC 10231 (IZ 9-11 mm), whose values were lower than our results. Shehata et al. (2020) performed an antimicrobial test of propolis ethanolic extracts from Egypt, Saudi Arabia, Oman, China, Bulgaria and Brazil against three Gram-positive, two Gram-negative bacteria, and four fungal strains. The authors found that Egyptian propolis was most active against *Bacillus cereus* ATCC 49064 (IZ 23.3 mm), *Salmonella senftenberg* ATCC 8400 (IZ 20.5 mm), *Aspergillus parasiticus* ITEM 11 (IZ 15.43 mm), *Fusarium oxysporum* ITEM 12591 (IZ 13.23 mm) and *C. albicans* ATCC MYA-2876 (IZ 14.43 mm). The propolis from Oman showed the highest inhibitory activity against *L. innocua* ATCC 33090 (IZ 15.4 mm). Chinese propolis was most active against *E. coli* BA 12296 (IZ 21.5 mm), *L. monocytogenes* ATCC 19116 (IZ 18.46 mm) and *A. flavus* ITEM 698 (IZ 20.93 mm). According to the same study, the Brazilian and Bulgarian propolis extracts demonstrated the lowest antimicrobial potential, as the Bulgarian sample failed to inhibit *L. monocytogenes* ATCC 19116, *A. parasiticus* ITEM 11 and *F. oxysporum* ITEM 12591.

In conclusion, the studied eighty Bulgarian propolis samples exhibited similar physicochemical properties (colour, pH and moisture content), but variable amounts of polyphenols and flavonoids expressed in the differences in their antioxidant and antimicrobial activities. Most of the tested propolis samples showed high levels of TPC and TFC and demonstrated significant antioxidant potential and high antimicrobial activity. Based on these estimations, we can summarize that Bulgarian propolis is a high quality bee product, which can find successful application as a remedy, a food additive improving the biological activity of the product, or a natural preservative extending the shelf-life and increasing the safety of various foods or cosmetic preparations. The future directions of this research will be related to additional studies of the chemical composition of the propolis samples, some other biological properties as antiviral activity and studies of the propolis applications in foods.

Conflict of interest

The authors declare no conflict of interests.

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