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Profiling of Turkish propolis subtypes: comparative evaluation of their phytochemical compositions, antioxidant and antimicrobial activities

Petar Ristivojević[1,2¶*], Ivica Dimkić[3¶*], Etil Guzelmeric[4], Jelena Trifković[5], Magdalena Knežević[6], Tanja Berić[3], Erdem Yesilada[4], Dušanka Milojković-Opsenica[5], Slaviša Stanković[3]

[1]University of Belgrade - Innovation Centre of the Faculty of Chemistry Ltd., Studentski trg 12-16, 11000 Belgrade, Serbia

[2]Ghent University Global Campus, Incheon, South Korea

[3]University of Belgrade - Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia

[4]Yeditepe University - Faculty of Pharmacy, Department of Pharmacognosy and Phytotherapy,
Kayisdagi Cad., Atasehir, 34755, Istanbul, Turkey

[5]University of Belgrade - Faculty of Chemistry, P.O. Box 51, 11158 Belgrade, Serbia

[6]Institute of Soil Science, Teodora Drajzera 7, 11000 Belgrade, Serbia

¶These authors contributed equally to this work.

*Corresponding authors:

Ivica Dimkić, PhD
Research associate
University of Belgrade - Faculty of Biology,
Studentski trg 16, 11000 Belgrade, Serbia
E-mail: ivicad@bio.bg.ac.rs

Petar Ristivojević, PhD
Research associate
University of Belgrade - Innovation Centre of the Faculty of Chemistry Ltd.,
Studentski trg 12-16, 11000 Belgrade, Serbia
E-mail: ristivojevic@chem.bg.ac.rs

Abstract

Comprehensive analysis of phenolic profiles of botanically different subtypes of Turkish propolis samples were performed using UHPLC–LTQ/Orbitrap/MS/MS method, and additionally total phenolic (TPC) and total flavonoid contents (TFC) as well as their antioxidative activities were evaluated by spectrophotometry. Antimicrobial activity of Turkish propolis against oral cavity bacteria from the genus *Streptococcus* (*S. pyogenes*, *S. sanguinis*, *S. mutans*) and *Candida albicans* ATCC 10231 was determined by diffusion and microdilution methods. Extensive fingerprint analysis of Turkish propolis revealed the presence of fifty one phenolic compounds, with fifteen quantified which confirm their affiliation to the two subtypes of the European propolis. All analysed samples have shown antimicrobial potential against all tested bacteria, with *S. pyogenes* being the most sensitive one. Turkish propolis, especially its orange subtype, can be considered as the high-quality product due to its rich phenolic and flavonoid content, strong antioxidative and antimicrobial activities. Turkish propolis could be, therefore, a good raw material for food and pharmaceutical industry.

Keywords: Phenolic profile of three subtypes of Turkish propolis; UHPLC–LTQ/Orbitrap/MS/MS; Total phenolic and flavonoid content; Antioxidant activity; Antimicrobial activity.

1. Introduction

Propolis is a natural resinous substance collected by honeybees (*Apis mellifera* L.) from different plant parts such as buds, branches, leaves and exudates (Yesilada, 2015). To date, two subtypes of propolis originated from *Populus* spp. were identified from Romanian, German, Serbian, Croatian, Slovenian and French propolis samples using several analytical techniques in combination with multivariate data analysis by various authors (Andjelković et al. 2017; Berthrams, Müller, Kunz, Kammerer, & Stintzing, 2013; Chasset, Häbe, Ristivojević, & Morlock, 2016; Morlock, Ristivojević, & Chernetsova, 2014; Milojković-Opsenica et al., 2016; Ristivojević et al. 2014; Sârbu, & Moţ, 2011). These authors suggested that all poplar type propolis samples could be categorized under two botanically different varieties known as orange (O) and blue (B) subtypes depending upon the color of the separated compounds on HPTLC plate under UV-light after derivatization. In addition to these findings, Guzelmeric et al. (2018) have confirmed the existence of O- and B-subtypes of propolis from Turkey, as well as the existence of a new subtype which was mainly composed of non-phenolic components. Previous studies on Turkish propolis samples have reported their chemical compositions and several biological effects (antimicrobial and antioxidant), while in these studies the authors have mainly focused on the geographical origin without identification of the plants constituents (Keskin, Hazir, Baser, & Kürkçüoglu, 2001; Koru et al., 2007; Uzel et al., 2005). However, botanical origin of propolis is an important task due to the fact that its chemical composition depends on the plant resource. Till now, mainly microscopic pollen analysis was applied to justify the botanical origin of Turkish propolis (Çelemli, & Sorkun, 2012). Gas Chromatography-Mass Spectrometry (GC-MS) was also used by several authors for investigation of chemical composition and determination of botanical origin of Turkish propolis (Duran et al., 2011). Furthermore, Popova, Silici, Kaftanoglu, & Bankova, 2005 investigated qualitative and quantitative

composition of Turkish propolis using TLC and GC-MS techniques and also determined its antibacterial activity. Botanical origins of propolis samples collected from different regions in Turkey were identified by simultaneous analysis of phenolic profile of propolis samples and plant buds' extracts by HPTLC, for the first time by our group (Guzelmeric et al., 2018). However, the phenolic composition of three subtypes of Turkish propolis, particularly based on its botanically different origins has not been investigated in detail so far.

Current paper is continuation of our previous research related to HPTLC phenolic profiles of Turkish propolis, authentication according to their botanical origins as well as determination of antioxidative activity (Guzelmeric et al., 2018). The main objective of the present study was the detailed phenolic profiling of O- and B-subtypes of Turkish poplar type propolis by ultrahigh-performance liquid chromatography (UHPLC) coupled with hybrid mass spectrometer, which combines the linear trap quadrupole (LTQ) and Orbitrap MS/MS mass analyser. In addition, the quality control parameters such as total phenolic content (TPC), total flavonoid content (TFC), as well as antioxidative activity and antimicrobial activity against oral cavity bacteria from the genus *Streptococcus* (*S. pyogenes*, *S. sanguinis*, *S. mutans*) and *Candida albicans* were also investigated. The results from this study might solve a question: Which subtype of Turkish propolis would be a better source of raw material for pharmaceutical and/or food industry?

2. Materials and methods

2.1. Chemical and materials

Methanol (HPLC grade), sodium carbonate, potassium chloride, Folin-Ciocalteu reagent, and filter paper (Whatman No.1) were purchased from Merck (Germany). 2,2-Diphenyl-1-picrylhydrazyl·(DPPH·) was purchased from Fluka AG (Switzerland). Ethanol (96 vol. %)

was purchased from J. T. Baker (Netherlands). Syringe filters (13 mm, PTFE membrane 0.45μm) were purchased from Supelco (USA). Ultrapure water was used in experiments (ThermoFisher TKA MicroPure water purification system, 0.055μS/cm). Aluminium chloride and standard phenolic compounds (chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, rutin, luteolin, quercetin, protocatechuic acid, *p*–hydroxybenzoic acid, cinnamic acid, apigenin, kaempferol, chrysin, pinocembrin, and galangin) were purchased from Sigma Aldrich (Germany). Streptomycin (stock 20 mg/mL), rifampicin (stock 100 mg/mL and 15μg/disc), ampicillin (stock 25 mg/mL), cefpodoxime (10 mg/disc), amphotericin B (100 units/disc), pristinamycin (15 mg/disc), clotrimazole (10 mg/disc), mezlocillin (75 mg/disc) and nystatin (stock 5 mg/mL) were purchased from Sigma Aldrich (Germany). Resazurin Sodium Salt (> 90% (LC) $C_{12}H_6NnaO_4$ = 251.17 g/mol) was purchased from TCI (Belgium).

2.2. Turkish propolis samples

In this study, forty-eight propolis samples [27 samples of orange, 17 of blue and the 4 of the third subtype propolis] (Guzelmeric et al., 2018), which were obtained from different regions of Turkey, were investigated (Fig. S1). Extraction procedure was described in our previous paper (Guzelmeric et al., 2018).

2.3. Measurement of the absorption spectra of propolis samples

The UV-Vis spectra were recorded using a Cintra 6 UV-Visible Spectrometer. Measurement of the absorption spectra was described in Ristivojević et al. (2017).

2.4. Estimation of the total phenolic content (TPC), total flavonoid content (TFC) and radical scavenging activity (RSA)

Total phenolic content (TPC), and total flavonoids content (TFC) were analysed according to Kumazawa et al. (2004). The 0.1 mL of EEP and 6.0 mL of deionized water were mixed with 0.5 mL of Folin-Ciocalteu reagent and the solution was incubated 5 min at room temperature. Then, 1.5 mL of sodium carbonate (20%) was added. After shaking and one hour of incubation at 40 ^{o}C, absorbance was measured at 760 nm. Gallic acid was used as a standard compound. The results were presented as mean value of three replicate measurements and expressed as mg of gallic acid equivalents (GAE) per gram of propolis sample.

For TFC, 0.5 mL of EEP was diluted with water up to 7.4 mL. Further, 0.4 mL of solution of aluminium chloride (10%) was added. Solution was shaken and incubated at room temperature for one hour; afterwards absorbance was measured at 420 nm. Quercetin was used as a standard. The results were presented as mean value of three replicate measurements and expressed as mg of quercetin (QE) per gram of propolis sample.

The radical scavenging activity (RSA**)** of the analysed samples was determined according to previous describes procedure (Ristivojević et al. (2017). The 0.1 mL of EEP and 4.0 mL of freshly prepared methanol solution of DPPH· (71 mM) were mixed and then left for 45 min in the dark. The reduction of the DPPH· radical was measured by monitoring continuously the decrease of absorption at 517 nm. RSA was calculated as a percentage of DPPH· discoloration using the equation:

$$RSA(\%) = \frac{\left(A_{DPPH} - A_{sample}\right)}{A_{DPPH}} \cdot 100$$

where A_{DPPH} is the absorbance of methanol solution of DPPH· radical, A_{sample} is the absorbance in the presence of propolis extract.

2.5. UHPLC–LTQ/Orbitrap/MS/MS

Qualitative and quantitative analysis as well as validation parameters of UHPLC–LTQ/Orbitrap/MS/MS method were described in our previous paper (Ristivojević et al., 2014). Chromatographic separations were performed using a UHPLC system consisting of a quaternary Accela 600 pump and Accela Autosampler (Thermo Fisher Scientific). An analytical Hypersil gold C18-column (50 × 2.1 mm, 1.9 μm particle size; Thermo Fisher Scientific) was used for separations. The mobile phase consisted of (A) water with 1% formic acid and (B) acetonitrile. The gradient programme was as follows: 0.0–10.0 min, 5–95% B; 10.0–12.0 min, 95% B; 12.0–12.2 min, 95–5% B; 12.2–15.0 min, 5% B. The injection volume for all samples was 5 μL and the flow rate was 300 μL/min. The UHPLC system was coupled to a linear ion trap and Orbitrap hybrid mass spectrometer (LTQ/Orbirrap) equipped with a heated- electrospray ionisation probe (HESI-II; Thermo Fisher Scientific). The mass spectrometer was operated in negative mode. Parameters of the ion source were as follows: source voltage 5 kV, capillary voltage –40 V, tube lens voltage –80 V, capillary temperature 275°C, sheath and auxiliary gas flow (N2) 42 and 11 (arbitrary units). The MS spectra were acquired by full-range acquisition covering 100–900 m/z. A data-dependant scan was performed for the fragmentation study by deploying collision- induced dissociation (CID). The normalised collision energy of the CID cell was set at 35 eV.

2.6. Bacterial strains and growth conditions

Antibacterial activity of all propolis samples was tested against *S. mutans, S. pyogenes and S. sanguinis* isolated from the human oral cavity (Nikolić et al., 2013) and against *Candida albicans* ATCC 10231. The Luria-Bertani (LB) medium (HiMedia, India) was used for culturing the bacterial strains, while TSB medium (Biomedics, Spain) was used for the growth of *C. albicans*. The number of viable cells (CFU/mL) was determined for each tested

strains at hourly intervals for a period of 8 hours. A single colony of the particular strain was inoculated in 150 mL of the appropriate growth medium in duplicate and shaked at 200 rpm and 37 °C. In parallel, optical density (OD) of the cultures was measured at 600 nm using a UV – 6300 PC double beam spectrophotometer (MRC, Israel)**.** The CFU/mL was obtained from appropriate dilutions which were plated onto LA and TSA agar plates in triplicate. For the each time interval, the growth curve was constructed and calibration was performed for each isolate. The microorganisms were grown to the optical density that matched to the 1×10^8 CFU/mL concentration of cells.

2.7. Diffusion assay

The initial screening of antimicrobial activity of all Turkish propolis samples was determined by well diffusion method as previously reported (Dimkić et al., 2016). Sterile molds for the wells were placed on the solid appropriate medium (LA and TSA) and 6 mL of LA/TSA soft agar inoculated with 60 μL (1×10^8 CFU/mL) of the appropriate strain added. Each of propolis samples was tested in three different concentrations (1, 0.5 and 0.25 mg/well) in two repetitions. The Petri dishes were incubated overnight at 37 °C. Antibiotic discs of cefpodoxime, amphotericin B, pristinamycin, clotrimazole, mezlocillin and rifampicin as well as ampicillin and streptomycin (0.2 and 0.4 mg/well) as an aqueous solution were used as a positive control for bacterial isolates and nystatin (0.1 and 0.15 mg/well) for *C. albicans*. As a negative control, 20 μL of methanol was used. The inhibition zone diameters were expressed in mm and graphically presented.

2.8. MIC assay

A broth microdilution method previously published (Ristivojević et al., 2016) was used to determine the minimum inhibitory (MIC), minimum bactericidal (MBC), and minimum

fungicidal concentration (MFC) for 39 selected propolis samples. Final concentration of each tested propolis sample in the first well was 1 mg/mL, while the concentration of methanol as a solvent was 10%. Two-fold serial dilutions of the propolis samples were made with LB and TSB media in 96-well microtiter plates. Besides a negative control (bacterial and fungal growth control), and a sterility control, the antibiotics streptomycin, rifampicin, ampicillin and nystatin were used as positive controls. The final concentration of antibiotics in the first well was 0.4 mg/mL. Each well, except for the sterility control, was inoculated with 20 μL of bacterial and fungal culture (1×10^8 CFU/mL), reaching a final volume of 200 μL. At the end, 22 μL of resazurin (oxidation-reduction indicator) was added to each well. The plates were incubated for 24 h at 37 °C. After incubation, the resazurin colour change reaction was observed. The MIC values were determined as no change in colour, while MBC and MFC were obtained by sub-culturing the test dilutions from each well without colour change on agar plates and incubating for 24 h. The lowest concentration that shows no bacterial growth was defined as the MBC value. The results were expressed in mg/mL.

2.9. Statistical analysis

The analysis of variance was supported by the Kolmogorov–Smirnov test for the normality of residuals and Levene's test for homogeneity of variance. The data obtained were subjected to analysis of variance (ANOVA) and means separation of MIC, MBC and MFC values, were accomplished by Tukey's HSD (honest significant difference) test. Significance was evaluated at $P < 0.05$. All dilutions were tested in duplicate with two repetitions.

Statistical analyses were conducted by the general procedures of STATISTICA v.7 (StatSoft, Inc.) and IBM SPSS Statistics v.20 (SPSS, Inc.).

3. Results and discussion

222 *3.1. Chemical profiling of propolis samples*

223 *3.1.1.UV/Vis spectroscopy*

224 The UV/Vis spectroscopy was applied to reveal the botanical origin of Turkish propolis, *i.e.*
225 to verify the presence of three botanically different subtypes. On the Fig. 1 differences in
226 UV/Vis patterns of O- and B-subtype propolis and specific profile of the third subtype are
227 indicated. The spectra of analysed samples showed characteristic UV/Vis pattern in the
228 regions between 200 to 400 nm with peaks attributable to the main classes of phenolics. O-
229 subtype propolis samples showed two absorption maximums at λ = 290 and 325 nm, B-
230 subtype at λ = 295 and 320 nm, while absorption maximum of the third subtype had low
231 intensity maximum at λ = 290 nm (Fig. 1). On the other hand, the UV/Vis absorption spectra
232 of Serbian O- subtype propolis were characterized with maximums at near λ = 270, 290 and
233 320 nm, while samples classified as B- subtype have two characteristic absorption maximums
234 at λ = 290 and 316 nm. Ristivojević et al. (2017) and Andjelković et al. (2017) also reported
235 UV/Vis spectra of two Serbian propolis subtypes and identified two main characteristic
236 absorption maximums at 291 nm and 314 nm. Same authors compared the UV/Vis spectra of
237 *Populus tremula*, and *P. x euramericana* with both Serbian propolis subtypes and identified
238 their botanical origins. UV/Vis spectra of Turkish propolis samples also showed
239 characteristic absorption bands similar to Serbian, Romanian, and Italian propolis samples
240 (Fabris, et al., 2013; Isla, Paredes-Guzman, Nieva-Moreno, Koo, & Park, 2005).
241 The three commonly applied assays of routine analysis of propolis are TFC, TPC and RSA. .
242 Orange subtype of propolis samples were characterized with higher mean value of TPC
243 (486.9 ± 184.2 mg/g) comparing to the B- subtype (310.6 ± 201.2 mg/g), while the lowest
244 TPC value was measured for the third subtype of propolis samples (115.7 ± 70.5 mg⁄g). Large
245 variations among data are not related only to the plant origin but also to the degree of

digestion by β-glycosidase from bees' saliva, and the percent of beeswax mixed with propolis. It is not unusual to get high variability among the data obtained from naturally occurring objects, i.e. samples. Turkish propolis showed much higher TPC values in comparison with the poplar subtype propolis of different geographic origins, *i.e.*, Chinese (Ahn et al., 2007), Japanese (Hamasaka, Kumazawa, Fujimoto, & Nakayama, 2007), and two times higher than Portugal (Moreira, Dias, Pereira, & Estevinho, 2008) samples. Above mentioned authors used maceration process of extraction with methanol and ethanol , while we in this study used ultrasonic extraction as a more efficient technique which could significantly influence on TPC and TFC values. Similar to TPC values, the O- subtype (265.7 ± 140.4 mg/g) samples have higher average TFC value in relation to B- subtype samples (185.5 ± 131.4 mg/g), and that of the third subtype of propolis (109.53 ± 54.42 mg/g). The flavonoids content was much higher comparing to Japanese (Hamasaka et al., 2007), Chinese (Ahn et al., 2007) and Serbian propolis (Ristivojević et al., 2017).

From the viewpoint of determined specifications with regard to phenolic compounds and flavonoids, Turkish poplar propolis may be considered as high quality propolis.

3.1.2.UHPLC–LTQ/Orbitrap/MS/MS

The qualitative and quantitative profile of phenolics was determined using the UHPLC system coupled to a LTQ OrbiTrap mass analyzer. UHPLC chromatograms of three subtypes of Turkish propolis were presented in Fig. 2. Fifteen phenolic compounds were quantified (Table 1). In all samples of Turkish propolis two benzoic acids derivatives (compounds **1** and **2)**, five phenolic acids (compounds **3-7)** and several flavanols (compounds **10**, **12** and **15**), flavones (compounds **9**, **11** and **13**), flavanones (compound **14**) and glycosides (compound **8**) were determined (Table 1). The concentration of almost all above mentioned compounds were higher in O-subtype of propolis comparing to other two subtypes (Table 1).

Compounds **1** and **2** as benzoic acids derivatives yielded two characteristic fragments at *m/z* 93 and *m/z* 109 by elimination of CO_2 and CH_3 groups from the molecule. The phenolic acids and their derivatives (compounds **3**–**16**) share a common fragmentation pathway based on loss of the CO_2 group resulting in $[M-H-CO_2]^-$, –44Da (Ristivojević et al., 2014). Compounds **7** and **8** were tentatively identified with specific fragmentation loss of CO_2 and CH_3, respectively. Caffeic acid and its derivatives (compounds **9**, **11-13**, **15, 16**) showed characteristic fragments at *m/z* 179, 161, and 135 (Table 2). Furthermore, *p*-coumaric acid derivatives (compounds **10** and **14**) produce ions at *m/z* 163 and 119, corresponding to *p*-coumaric acid and the fragment obtained after loss of CO_2. Compound **10** showed several more characteristic fragments at *m/z* 295, 277, 191, 179, 163, 135, 119; it was identified in both Turkish propolis subtypes (Kečkeš et al., 2013). Compounds **5** and **7**were identified as main phenolic components in orange and blue subtypes of Turkish propolis.

Using LTQ-Orbitrap-MS^2 analysis, the comprehensive fragmentation pathways of flavonoids were identified, while ten compounds were additionally quantified (Table 2). Nine flavonols identified in Turkish propolis shared common fragmentation pathway of flavonols that correspond to retro-Diels–Alder (RDA) reaction (Kečkeš et al., 2013). Compounds **22** and **23** produce two common ions at *m/z* 315 and 299. Additionally, in case of compound **18** and compound **20** ion at *m/z* 300 was attributed to $[M–H–CH_3]^-$ (Ristivojević et al., 2014). Flavonols such as compounds **17**, **19** and **24** were recognized by several authors as markers of O-subtype of propolis from France, Germany, Serbia, and Turkey (Ristivojević et al., 2014). Based on the HPTLC fingerprinting of Turkish propolis samples analysed in our previous study (Guzelmeric et al., 2018), these phenols showed orange bands characteristic for O-subtype propolis. Compounds **17** and **24** were found in O-subtype propolis in higher amount (Table 1). Compound **25** produced several fragments at *m/z* 257, 242, 199, and 125, confirmed by literature data (Leveques et al., 2012; Mišić et al., 2015).

Mass spectra of Turkish propolis samples indicated seven flavanonols and their esters and ethers (Table 2). Compound **26** and its derivatives (**26**-**32**) were characterised by the same fragments obtained by loss of the acyl group, yielding ions at *m/z* 271 and 253, which correspond to $[M–acyl]^-$ and $[M–acyl–H_2O]^-$, respectively (Kečkeš et al., 2013).

Five flavones (compounds **33**-**37**) were identified with two commonly ions such as *m/z* 117 and 151, which corresponded to the RDA fragmentation pathway. Compound **36** showed ions at *m/z* 209, 181, and 143 which correspond to $[M–H–CO_2]^-$, $[M–H–CO_2–CO]^-$, $[M–H–C_3O_2–C_2H_2O]^-$. Compounds **36**, together with **24** and **42** were found in O- subtype in higher amount than in blue and the third subtypes (Table 1). Compounds **36** was also identified as a characteristic component of O- subtype propolis from Turkey with a green band on the HPTLC chromatogram (Guzelmeric et al., 2018) in higher concentration comparing to other two subtypes (Table 1). Fragment ions, $[^{1,3}A]^-$, $[^{1,3}A–CO_2]^-$ and $[^{1,3}B]^-$ were identified for compound **34** (Kečkeš et al., 2013; Ristivojević et al., 2014). The molecular ion of **37** produced fragment ion at *m/z* 117, possibly originated from $[^{1,3}B]^-$. Compounds **33** and **35** showed a fragment at *m/z* 151; these flavonoids were also identified in Serbian and German propolis samples (Kečkeš et al., 2013; Morlock et al., 2014).

Examination of mass spectra of propolis samples revealed that there are six flavanone derivatives in the Turkish propolis samples (compounds **38**-**42**) based on the peaks of fragmentation ions $[^{1,3}A]^-$ and $[^{1,3}B]^-$. Pinocembrin and pinobanksin were reported to be the main components for poplar type propolis (Ristivojević et al., 2014). Compounds **41** and **42** produced characteristic fragments at *m/z* 254 and 213 originated by loss of CH_3 and C_2H_2O groups, respectively, as previously described in the literature (Kečkeš et al., 2013). Compounds **38**, **39**, and **40** yielded characteristic fragments at *m/z* 119, which were found in both orange and blue subtypes of Turkish propolis (Table 2) (Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001; Ristivojević et al., 2014). As we mentioned in our previous reports,

galangin, pinocembrin, chrysin, kaempferol, quercetin, caffeic acid, caffeic acid phenethyl ester (CAPE), luteolin and apigenin were the main components of O- subtype of Serbian and Turkish propolis samples (Table 1) (Guzelmeric et al., 2018; Ristivojević et al., 2014).

Recently, the presence of flavonoid glycosides in Portuguese and Serbian propolis samples, although the number of such reports were quite few (Falcão et al., 2001; Ristivojević et al., 2014). In the present paper, presence of three glycosides such as compounds **43**, **44**, **45** were identified in Turkish propolis. Rutin was quantified in B- subtype propolis in higher amount compared to O- subtype; two ions at *m/z* 315 and *m/z* 300 were formed as a result of elimination of rutinoside and rutinoside–CH_3 units, respectively (Falcão et al., 2013; Ristivojević et al., 2014). Same fragments were also identified in compound **45** with a molecular ion peak at *m/z* 463.0848. Compound **44** was quantified in higher amount in O- subtype propolis and characterized by a typical fragmentation pattern with three ions at *m/z* 269, 268, and 151.

Phenolic glycerides were found in North Russian, Bulgarian, Swiss, German, Russian, Polish, Belarusian, Croatian, Serbian as well as Turkish propolis samples and they probably originated from various *Populus* hybrids (Bankova, Popova, Bogdanov, & Sabatini, 2002; Bertrams et al., 2013; Falcão et al., 2013;. Isidorov, Szczepaniak, & Bakier,, 2014). On the other hand, seven phenolic glycerides were identified in Turkish propolis samples. Compound **46** and **47** formed a fragment ion at *m/z* 179 originating from caffeic acid, which is in accordance with literature data (Svensson et al., 2010). Furthermore, compounds **48**-**51** had fragments at *m/z* 193, 179, 163, and 161 (Table 2), which could be inferred as *p*-coumaric acid, caffeic acid and ferulic acid esterified to glycerol (Ristivojević et al., 2014).

3.2. Biological profile of Turkish propolis samples

3.2.1. Antioxidative activity

Antioxidant capacity of propolis samples was determined by radical scavenging activity. The average RSA value of Turkish propolis samples was 55.01 ± 27.23%. Samples of O- subtype exerted higher RSA value (65.64 ± 25.88%) in comparison with the B-subtype (42.22 ± 24.42%) as well as the third subtype of propolis (26.49 ± 6.72%) (Fig. S2). Higher RSA value of O- subtype propolis might possibly correlate with higher TPC and TFC values. These results are in accordance with our previous findings evaluated by HPTLC-DPPH· assay (Guzelmeric et al., 2018). The RSA values of Chinese (Ahn et al., 2007) and Serbian types (Ristivojević et al. 2017) were almost identical, while that of Japanese type was significantly lower (Hamasaka et al., 2004). In our previous study, we identified potential antioxidative components such as caffeic acid, CAPE, pinobanksin and galangin in both propolis subtypes (Guzelmeric et al., 2018).

3.2.2. Antimicrobial assays

Before assaying antimicrobial activity, the growth conditions of each strain were determined. The growth curves were constructed (Fig. S3), based on obtained data from repeated experiments (Table S1). According to the calibration curves, optical densities which corresponded to the 1×10^8 CFU/mL were determined: 0.30, 0.12, 0.15 and 1.52 for strains *S. mutans*, *S. pyogenes*, *S. sanguinis* and *C. albicans*, respectively.

3.2.2.1. Diffusion assay

According to the obtained results, *S. sanguinis* was the most resistant strain against all tested propolis samples. The O- subtype propolis samples showed moderate activity exclusively at highest concentration against this strain, while B- and the third subtypes of propolis samples mostly exerted no antibacterial activity against this strain (Fig. 3 and 4). The reference antibiotic mezlocillin demonstrated a potent antimicrobial activity against *S. sanguinis*, with 31 mm of inhibition zone, while streptomycin and rifampicin showed moderate activity

against this pathogen (16 and 13 mm). Other tested antibiotics had no effect against *S. sanguinis.*

Turkish propolis samples showed moderate antibacterial activities against *S. mutans and C. albicans* strains, while eleven and fifteen propolis samples had no activity against these strains, respectively. Some O- and B- subtypes of propolis produced inhibition zones larger than 12 mm, at 0.5 mg/well concentration. In general, *S. mutans and C. albicans* were more sensitive to the O- subtype. These samples also had the highest values for TPC. Among the reference antibiotics streptomycin and mezlocillin showed the strongest activity against *S. mutans* (25 mm), while rifampicin produced smaller inhibition zone (17 mm). Other antibiotics, except pristinamycin with the smallest inhibition zone diameter, showed no antibacterial effect against this strain. Nystatin showed weaker antifungal activity against *C. albicans*, comparing to the many of the tested propolis samples.

Among the tested microorganisms, *S. pyogenes* was the most sensitive strain. Samples of the third propolis subtype had antibacterial effect only against this strain (Fig. 4). Almost all tested propolis samples produced inhibition zones at 1 mg/well concentration. In general, samples of O- subtype propolis exerted a higher antimicrobial activity. Rifampicin demonstrated the highest antibacterial effect against *S. pyogenes*, with 27 mm of inhibition zone diameter. Amphotericin B and ampicillin had no effect against this strain, while all other antibiotics showed moderate activity (10-17 mm). Out of all tested samples, the sample 8 had the strongest activity against *S. pyogenes* and *S. mutans*. Sample 40 had the strongest activity against *S. sanguinis*, and samples 24 and 25 against *C. albicans*. Samples 40, which possess a lower TPC value, had the best activity against resistant *S. sanguinis* strain. Higher flavonoid content might be responsible for the potential bacterial activity.

3.2.2.2. MIC assay

MIC, MBC and MFC values were determined for the 39 propolis samples (24 samples O-, 14 samples B- and one of the third subtypes) based on well diffusion assay results. MIC values for the most samples were found in the concentration range from 0.01 to 1 mg/mL (Table 3). Sample 18 was the only one showing the strongest activity against all strains, with MIC values lower than 0.10 mg/mL. The majority of O- subtype of propolis samples exerted a strong antimicrobial activity against various strains, often with MIC values lower than 0.10 mg/mL. The third subtype propolis sample (30) exerted a higher antimicrobial effect against *S. pyogenes* (0.14 mg/mL), while a weak activity against *C. albicans* (1 mg/mL). Similar results were also observed in diffusion test. Also TPC, TFC and RSA values were low for this sample, while cinnamic acid was the main component. MIC values against *S. sanguinis* were ranging from 0.06 mg/mL (sample 18) to over 1 mg/mL for the sample 45 which had also low TPC and TFC values. Like in diffusion assay, *S. sanguinis* was the most resistant strain in this assay. Higher MIC values (0.50 - 1 mg/mL) were recorded for several O- and B-subtypes of propolis (2, 28, 31, 32, 33, 34, 35, 37, 38, 39, 40, 41, 43 and 45). Among these samples 37, 38 and 43 were found to contain high concentration of cinnamic acid, in addition to ferulic and caffeic acids as the main components, while sample 2 was found to be rich in chlorogenic acid (around 50 times higher than in the others). Other propolis samples had MIC values lower than 0.50 mg/mL. MIC values were ranged for B- subtypes of propolis samples against *S. mutans* from 0.03 (sample 3) to 0.75 mg/mL (sample 45). On the other hand, the lowest MIC values (less than 0.1 mg/mL) were recorded for O- subtype samples (8, 18, 22, 28, 29, 33 and 35). Samples 8, 18, 28 and 29 showed to possess strong activity against this strain in diffusion assay. Sample 3 had extremely high TPC, TFC and RSA values. Except caffeic and ferulic acids, *p*-coumaric acid was also presented in a higher concentration in samples 18 and 28. In general, all tested samples, except samples 15, 40 and 45, had MIC values lower than 0.50 mg/mL. MIC values against *S. pyogenes* were ranging from 0.01

(sample 18) to 1 mg/mL (sample 40). *Streptococcus pyogenes* was the most sensitive strain, with the lowest MIC values ranging from 0.01 to 0.09 mg/mL, against the most of O- subtype samples. Sample 30 (the third subtype) also had low MIC value against this strain which was in accordance with the diffusion assay results. MIC values against *C. albicans* ranged from 0.06 to over 1 mg/mL. The O- subtype sample **2**, and B- subtype samples 40 and 45, had the highest MIC values and absence of antifungal activity in diffusion assay. A few O- subtype samples (11, 18, 22 and 25) had the lowest MIC values ranging between 0.06 - 0.09 mg/mL, while some others (2, 4, 7, 17, 5, 20, 30, 37, 40, 43 and 45) had the highest MIC values. The rest of the samples had shown medium MIC values, less than 0.5 mg/mL. For samples 2, 30, 40, 41, 43, and 45 MBC/MFC were not determined (MBC/MFC > 1 mg/mL) against particular strains. In general, MBC values were twice and even three times higher than the MIC values (Table 4) for the most of the samples. The majority of samples had two times higher MFC than MIC values against *C. albicans.* MFC values for samples 2, 30, 40 and 45 were not found at all, while MBC values for samples 40, 43, and 45 were at 1 mg/mL or higher. Methanol as solvent did not show any antimicrobial activity. All three tested bacterial strains exerted resistance against ampicillin, and also *S. pyogenes* against streptomycin. Rifampicin had a lowest MIC value against *S. pyogenes* (0.006 mg/mL), while higher values were recorded against *S. mutans* (0.1 mg/mL) and *S. sanguinis* (0.2 mg/mL). Streptomycin showed highest inhibitory rates against *S. sanguinis* and *S. mutans* (0.025 mg/mL). On the other hand, MIC value of nystatin against *C. albicans* was 0.4 mg/mL, which was significantly higher than for all propolis samples.

3.2.2.3. General observations

Only a few studies have investigated the antimicrobial potential of Turkish propolis. Oral use of propolis as the most common form of application, or in the form of vaginal tablets, provides an incentive in finding adequate propolis samples as an alternative for the control of

selected opportunistic and pathogenic microorganisms tested in this study. *Candida albicans* is an opportunistic pathogen, which exists in several morphological forms. In case of immunity collapse, this type of over expression occurs, causing a candidiasis disease that may be oropharyngeal, vulvovaginal or invasive (Sudbery, Gow, & Berman, 2004). The presence of *Streptococcus mutans* in the oral cavity is associated with the formation of caries, gingivitis and chronic periodontitis (Contardo, Díaz, Lobos, Padilla, & Giacaman, 2007). *Streptococcus sanguinis* is the most common bacterial causative agent of the dental plaque, and its presence in combination with *S. mutans* is also associated with the formation of caries and other diseases of the tooth (Borges, Ferreira, Saavedra, & Simões, 2013). *Streptococcus pyogenes* is a trigger of pharyngitis, which most commonly occurs in inflammatory mucous membranes of the nasal and sinus, oral cavity and tonsils (Lyon, & Caparon, 2003). The results of antimicrobial activity of Turkish propolis against particular oral microorganisms, used in this study, are scarce. In one of these studies, a good antimicrobial activity of propolis samples from Central Anatolia was obtained with an average concentration of 0.1 mg/mL against *S. mutans* (Arslan, Silici, Percin, Koç, & Er, 2012). Similarly antimicrobial activity of propolis samples from two different areas in Marmara region of Turkey have been reported against the beta-hemolytic streptococci by Keskin et al. (2001).

Otherwise, antimicrobial effects of various propolis types from other parts of the world have been investigated by several research groups. Australian propolis showed very strong antibacterial activity against *Streptococcus* isolates (Nam et al., 2016), while Nigerian propolis demonstrated potent activity against *S. mutans* (Ophori et al., 2010). The average inhibition zone of Nigerian propolis was high (24 mm), which is considerably higher than that of the Turkish propolis (9.3 mm). In another study Iraqi propolis showed activity against *S. pyogenes* (Hendi, Naher, & Al-Charrakh, 2010) with a similar inhibition zone as it was observed in the present study.

On the other hand, *C. albicans* was found to be resistant to the Iraqi and Serbian propolis samples (Hendi et al., 2010; Stepanović et al., 2003), while a moderate activity was determined by the Lebanese propolis samples (Chamandi et al., 2015). Hegazi et al. (2000) also reported that *C. albicans* isolates were found to be quite resistant to propolis, with MIC values higher than 1 mg/mL, while propolis samples from the Mediterranean part of Turkey showed a moderate activity against *C. albicans* (Velikova et al., 2000). A similar antifungal activity profile has been reported for propolis samples from the other parts of Turkey (Katircioglu, & Mercan, 2006).

In the present study, many of the samples originating from Eastern Anatolia (18 samples) showed strong or moderate antimicrobial activity against different isolates. More samples that had similar antimicrobial potential were provided from other regions of Turkey: Marmara (8 samples), Mediterranean (4 samples), Aegean (3 samples), Black Sea (4 samples) and South eastern Anatolia (1 sample). However, the sample 18 showed the strongest activity against all tested strains which was comparable with the activity of streptomycin. This sample also had an extremely high TPC and TFC values, while caffeic and ferulic acids were determined as the main constituents. We cannot mark more propolis samples which exhibited equally strong antimicrobial activity against all isolates. The cinnamic acid concentration was the highest among all tested samples. Ferulic and caffeic acid were also present in almost all samples with strong antimicrobial activity; these compounds might possibly contribute to the antimicrobial activity of propolis samples. As a matter of fact, cinnamic, chlorogenic and *p*-coumaric acids were also quantified in higher concentrations in several samples with strong antimicrobial activity. According to the previous reports, ferulic (Borges, Ferreira, Saavedra, & Simões, 2013) and caffeic acids (Mirzoeva, Grishanin, & Calder, 1997) exerted their antimicrobial effects on the cell membrane, inducing irreversible changes and damage.

Accordingly, it is evident that phenolic acids exert higher contribution to the antimicrobial activity of Turkish propolis samples than flavonoids.

4. Conclusions

Recently, demand for propolis on the market has steadily increasing due to its evidenced health benefits. However, some propolis products are marketed without examining their chemical compositions, without identifying the plant sources or determining the type of propolis. On the other hand, in case when honeybees cannot find possible plant sources around, they may collect materials such as paint, asphalt and/or mineral oils which would raise the risk for the human health when consumed due to such toxic contamination and also reduced the pharmacological effects. For this reason, it is extremely important to analyse the quality, to determine the chemical composition and the botanical origin of propolis, which would have direct impact on its health benefits or risks.

In this study, the phenolic profiles of Turkish propolis samples from different botanical origins were evaluated in detail. Moreover, TPC, TFC, antioxidant and antimicrobial potentials were determined of O-, and B- as well as the third subtypes of Turkish propolis. Experimental results have shown that particularly O-subtype of propolis originated mainly from *Populus nigra* could be used as a raw material in pharmaceutical and/or food industry due to its rich phytochemical composition and a wide range of health benefits.

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Figure Captions

Fig. 1. UV/Vis spectra of three subtypes of Turkish propolis (A- Orange type, B- Blue type, C-Third type).

Fig. 2. Total ion chromatograms (TICs) of three subtypes of Turkish propolis samples, obtained with the LTQ-Orbitrap XL instrument in negative ion mode (A, B- Orange type, C-Third type, D-Blue type).

Fig. 3. Antimicrobial potential of the orange subtype samples of Turkish propolis tested by diffusion method at concentrations of 1 (A), 0.5 (B) and 0.25 mg/well (C).

Amp - Ampicillin, Stp - Streptomycin, Rif - Rifampicin, Mez - Mezlocillin, Klo - Clotrimazole, Pri - Pristinamycin, Cef - Cefpodoxime, Amf B - Amphotericin B, and Nys – Nystatin.

Fig. 4. Antimicrobial potential of the blue and third (in rectangles) subtypes samples of Turkish propolis tested by diffusion method at concentrations of 1 (A), 0.5 (B) and 0.25 mg/well (C).

Amp - Ampicillin, Stp - Streptomycin, Rif - Rifampicin, Mez - Mezlocillin, Klo - Clotrimazole, Pri - Pristinamycin, Cef - Cefpodoxime, Amf B - Amphotericin B, and Nys – Nystatin.

Table Captions

Table 1. The content of phenolic compounds (expressed in mg/mL as mean ± SD) in three subtypes of Turkish propolis.

Table 2. Phenolic compounds tentatively identified in Turkish propolis.

Table 3. The minimum inhibitory concentration (MIC) of Turkish propolis samples (mg/mL). The mean values and standard error are shown.

Table 4. The minimum bactericidal (MBC) and fungicidal concentrations (MFC) of Turkish propolis samples (mg/mL). The mean values and standard error are shown.

Table 1. The content of phenolic compounds (expressed in mg/mL as mean ± SD) in three subtypes of Turkish propolis

No.	Phenolic compounds	Orange type	Blue type	Third type
1	*p*-Hydroxybenzoic acid	2.24 ± 1.74	1.44 ± 1.17	0.46 ± 0.23
2	Vanillic acid	0.39 ± 0.26	0.30 ± 0.15	0.27 ± 0.11
3	Protocatechuic acid	1.69 ± 1.01	0.71 ± 0.24	0.45 ± 0.19
4	Caffeic acid	34.78 ± 16.77	24.82 ± 18.70	3.96 ± 1.93
5	*p*-Coumaric acid	4.91 ± 3.69	3.13 ± 2.25	0.19 ± 0.11
6	Cinnamic acid	5.19 ± 4.67	3.00 ± 2.24	5.28 ± 4.21
7	Ferulic acid	19.42 ± 18.38	9.63 ± 5.91	1.00 ± 0.63
8	Rutin	0.36 ± 0.17	0.47 ± 0.32	0.16 ± 0.09
9	Luteolin	1.57 ± 0.87	1.24 ± 0.74	0.31 ± 0.18
10	Quercetin	4.33 ± 1.56	2.85 ± 1.44	1.11 ± 0.75
11	Apigenin	1.56 ± 0.64	1.05 ± 0.43	0.54 ± 0.32
12	Kaempferol	1.76 ± 0.72	0.92 ± 0.45	0.44 ± 0.29
13	Chrysin	2.22 ± 0.89	1.85 ± 0.56	1.54 ± 0.86
14	Pinocembrin	2.81 ± 1.00	2.16 ± 0.84	0.94 ± 0.37
15	Galangin	2.70 ± 1.39	1.67 ± 0.40	0.96 ± 0.51

Table 2. Phenolic compounds tentatively identified in Turkish propolis

No.	Identified compounds	t_R (min)	Calculated mass $[M-H]^-$	Accurate mass $[M-H]^-$	Error (ppm)	Fragmentation	Reference
	Benzoic acid and its derivatives						
1	*p*-Hydroxybenzoic acid	5.19	137.02442	137.02230	2.12	109, 93	Natić et al., 2015
2	Vanillin	6.55	151.04007	151.03960	0.47	136	
	Phenolic acids and their derivatives						
3	Protocatechuic acid	4.07	153.01970	153.01800	1.7	136 [M-H-H_2O], 109, [M−H−CO_2]$^-$, 107	Kečkeš et al., 2013
4	Protocatechuic acid or is isomer	5.02	153.0197	153.0183	1.4	136 [M-H-H_2O], 109 [M−H−CO_2]$^-$	Kečkeš et al., 2014
5	Caffeic acid	5.18	179.035	179.0336	1.4	161[M-H-H_2O] , 151, 135 [M−H−CO_2]$^-$	Kečkeš et al., 2013, Pellati et al., 2011
6	*p*−Coumaric acid	6.49	163.0401	163.0387	1.4	119 [M−H−CO_2]$^-$	Kečkeš et al., 2013, Pellati et al., 2011
7	Ferulic acid	6.73	193.0506	193.0495	1.1	179 [M−H–CH_3]$^-$, 178, 149 [M-H-CH_3-CO_2]$^-$, 134	Kečkeš et al., 2013, Pellati et al., 2011
8	Cinnamic acid	8.55	147.0452	147.0449	0.3	103 [M−H−CO_2]$^-$	Kečkeš et al., 2013
9	3,4-Dimethyl-caffeic acid (DMCA)	8.16	207.0663	207.0645	1.8	179 [M-H-2CH_3]$^-$, 163 [M-H-CO_2]$^-$	Pellati et al., 2011
10	*p*−Coumaroylquinic acid	9.07	337.0929	337.0912	1.7	295, 277, 179, 191 [$C_7H_{11}O_6$]-,161, 135, 119	Weisz et al., 2009
11	Prenyl caffeate	11.26	247.0976	247.0972	0.4	179 [$C_9H_7O_4$]$^-$, 135 [$C_9H_7O_4$-CO_2]$^-$	Gardana et al., 2007, Medana et al., 2008
12	Caffeic acid phenethyl ester (CAPE)	11.60	283.0976	283.0948	2.8	179 [$C_9H_7O_4$]$^-$, 135 [$C_9H_7O_4$-CO_2]$^-$	Kečkeš et al., 2013
13	Caffeic acid cinnamylester	12.19	295.0976	295.0956	2.0	179 [$C_9H_7O_4$]$^-$,135 [$C_9H_7O_4$-CO_2]$^-$	Pellati et al., 2011
14	*p*−Coumaric methyl butenyl ester	12.37	231.102	231.101	1.0	163 [$C_9H_7O_3$], 119 [M−H−CO_2]$^-$	Gardana et al., 2007
15	Benzyl caffeate	12.72	269.0819	269.0811	0.8	179 [$C_9H_7O_4$]$^-$, 135 [$C_9H_7O_4$-CO_2]$^-$	Gardana et al., 2007, Pellati et al., 2011
16	Methyl-*O*-caffeoylquinate	13.21	367.10346	367.10010	3.36	179, 161, 135	Natić et al., 2015
	Flavonols						
17	Quercetin	8.54	301.0354	301.0331	2.3	271, 179 [^{1,2}A]$^-$, 151 [^{1,2}A–CO]$^-$, 121 [^{1,2}B]$^-$	Kečkeš et al., 2013, Fabre et al., 2001
18	Rhamnetin	8.88	315.051	315.0486	2.4	300 [M–H–CH_3]$^-$	Kečkeš et al., 2013, Fabre et al., 2001
19	Kaempferol	8.90	285.0405	285.0395	1.0	267 [M−H−H_2O]$^-$, 241 [M–H–CO_2]$^-$, 199 [M–H–C_2H_2O–CO_2]$^-$, 151 [^{1,3}A]$^-$	Kečkeš et al., 2013
20	Isorhamnetin	8.96	315.051	315.0564	-5.4	300 [M–H–CH_3]$^-$, 151 [^{1,3}A]$^-$	Kečkeš et al., 2013, Fabre et al., 2001
21	Kaempferide	10.50	299.0561	299.054	2.1	284 [M–H–CH_3]$^-$,151 [^{1,3}A]$^-$	Kečkeš et al., 2013
22	Bis−methylated quercetin	10.59	329.0642	329.0642	0.0	315 [M–H–CH_3]$^-$, 299 [M–H–2CH_3]$^-$	Kečkeš et al., 2013
23	Bis−methylated quercetin	10.91	329.0667	329.0654	1.3	315 [M–H–CH_3]$^-$, 299 [M–H–2CH_3]$^-$	Kečkeš et al., 2013
24	Galangin	11.30	269.0456	269.0455	0.1	213 [M–H–C_2O_2]$^-$, 183 [M–H–C_2H_2O–CO_2]$^-$,151 [^{1,2}A–CO]$^-$	Kečkeš et al., 2013
25	Hesperetin	11.93	301.07176	301.06940		257, 242, 199, 125	Leveques et al., 2012
	Flavanonols						
26	Pinobanksin	9.02	271.0612	271.0593	1.9	253 [M–H–H_2O]$^-$, 243 [M–H–CO]$^-$,	Kečkeš et al., 2013, Pellati et al., 2011
27	Pinobanksin−5−methyl-ether−3−*O*−acetate	9.17	327.087	327.0851	1.9	285 [M−acetate]$^-$, 165 [M–H–acetate-H_2O–2CO_2]$^-$	Kečkeš et al., 2013, Pellati et al., 2011
28	Pinobanksin−3−*O*−acetate	11.67	313.0712	313.0686	2.6	271 [M-acetate]$^-$, 253 [M-acetate-H_2O]$^-$	Kečkeš et al., 2013
29	Pinobanksin−5−methyl-ether	11.83	285.0767	285.0749	1.8	271 [M–CH_3]$^-$, 253 [M–CH_3–H_2O]$^-$, 239 [M–H–H_2O–CO]$^-$,	Kečkeš et al., 2013, Pellati et al., 2011
30	Pinobanksin−3−*O*−propionate	12.17	327.0869	327.085	1.9	271 [M-propionate]$^-$, 253 [M-propionate-H_2O]$^-$	Kečkeš et al., 2013
31	Pinobanksin−3−*O*−butyrate (or isomer)	13.43	341.1002	341.106	-5.8	253 [M-H-butyrate-H_2O]$^-$	Kečkeš et al., 2013
32	Pinobanksin−3−*O*−pentanoate (or isomer)	14.20	355.1183	355.1228	-4.5	271 [M-H-pentanoate]$^-$, 253 [M-H-pentanoate-H_2O]$^-$	Kečkeš et al., 2013
	Flavones						
33	Luteolin	4.14	285.0405	285.0385	2.0	213 [M - H - CO_2 - CO]$^-$, 151 [^{1,3}A]$^-$,	Kečkeš et al., 2013
34	Apigenin	9.53	269.0456	269.0385	7.1	151 [^{1,4}B+2H]$^-$, 149 [^{1,4}B]$^-$, 117 [^{1,3}B]$^-$	Kečkeš et al., 2013
35	Acacetin	11.40	283.0612	283.0593	1.9	151, 107	Kečkeš et al., 2013
36	Chrysin	12.05	253.0506	253.0486	2.0	209 [M–H–CO_2]$^-$, 181 [M–H–CO_2–CO]$^-$, 143 [M–H–C_3O_2–C_2H_2O]$^-$	Kečkeš et al., 2013
37	Dihydroxyflavone	12.40	253.0506	253.0486	2.0	117 [^{1,3}B]$^-$	Kečkeš et al., 2013

	Flavanones						
38	Sakuranetin	11.87	285.0769	285.0749	2.0	165 $[C_8H_5O_4]^-$, 119	Kečkeš et al., 2013
39	Naringenin	11.96	271.0612	271.0601	1.1	151 $[^{1,3}B]^-$, 119 $[^{1,3}A]^-$	Fabre et al., 2001
40	Liquiritigenin	12.11	255.0663	255.0635	2.8	153 $[^{1,3}A]^-$, 135 $[^{1,3}A\text{-}H_2O]^-$, 119 $[^{1,3}A\text{-OH-OH}]^-$	Wang et al. 2008
41	Pinostrobin	12.18	269.0819	269.0797	2.2	254 $[M\text{-}H\text{-}CH_3]$, 165 $[^{1,3}A]^-$	Kečkeš et al., 2013
42	Pinocembrin	12.46	255.0663	255.0663		213 $[M\text{-}H\text{-}C_2H_2O]^-$, 151 $[^{1,3}A]^-$	Kečkeš et al., 2013
	Glycosides						
43	Rutin	6.23	609.1461	609.1443	1.8	301 [M–H–glycoside]$^-$, 300	Kečkeš et al., 2013
44	Apigetrin (Apigenin-7-*O*-glucoside)	6.69	431.0984	431.0959	2.5	269 [M–H–glycoside]$^-$, 268, 151 $[^{1,4}B\text{-}2H]^-$	Hossain et al., 2010
45	Quercetin 3-*O*-galactoside	6.88	463.08820	463.08480	3.4	301, 300	
	Phenolic glycerides						
46	Caffeoylglycerol	5.5	253.071	253.0702	0.8	179 $[C_9H_7O_4]^-$	Svensson et al., 2010
47	Coumaroylferuoyl glycerol	6.04	413.1212	413.1217	-0.5	235, 193 $[C_{10}H_9O_4]$-, 163 $[C10H_9O_4\text{-}2CH_3]$-	Ma et al., 2007
48	Dicoumaroyl acetyl glycerol	6.48	425.1224	425.1221	0.3	365, 321, 163 $[C_9H_7O_4]^-$	
49	Dicaffeoyl acetyl glycerol	9.55	457.1122	457.11	2.2	397, 295, 235, 179, 161	
50	Acetyl-coumaroyl--feruloylglycerol	10.58	425.1236	425.1216	2.0	263, 179, 161	
51	Acetyl-diferuloylglycerol	11.46	485.144	485.1421	1.9	425, 381, 207, 193	Shi et al., 2012

Table 3. The minimum inhibitory concentration (MIC) of Turkish propolis samples (mg/mL). The mean values and standard error are shown.

Subtype of propolis	Sample	MIC			
		S. sanguinis	***S. mutans***	***S. pyogenes***	***C. albicans***
O	2	$0.50^{abcd} \pm 0.00$	$0.15^{cde} \pm 0.05$	$0.02^{cd} \pm 0.04$	$> 1.00^{a} \pm 0.00$
O	4	$0.25^{bcd} \pm 0.00$	$0.37^{bcd} \pm 0.07$	$0.14^{bcd} \pm 0.06$	$0.50^{b} \pm 0.00$
O	7	$0.15^{bcd} \pm 0.05$	$0.25^{bcde} \pm 0.00$	$0.25^{bcd} \pm 0.00$	$0.50^{b} \pm 0.00$
O	8	$0.15^{bcd} \pm 0.03$	$0.09^{cde} \pm 0.01$	$0.13^{bcd} \pm 0.06$	$0.25^{cde} \pm 0.00$
O	11	$0.17^{bcd} \pm 0.04$	$0.28^{bcde} \pm 0.12$	$0.14^{bcd} \pm 0.05$	$0.09^{de} \pm 0.01$
O	12	$0.12^{cd} \pm 0.00$	$0.18^{bcde} \pm 0.03$	$0.07^{cd} \pm 0.02$	$0.25^{cde} \pm 0.00$
O	16	$0.25^{bcd} \pm 0.00$	$0.37^{bcd} \pm 0.07$	$0.28^{bcd} \pm 0.12$	$0.31^{bcd} \pm 0.10$
O	17	$0.12^{cd} \pm 0.00$	$0.34^{bcde} \pm 0.09$	$0.08^{cd} \pm 0.02$	$0.50^{b} \pm 0.00$
O	18	$0.06^{d} \pm 0.00$	$0.09^{cde} \pm 0.01$	$0.01^{d} \pm 0.00$	$0.06^{e} \pm 0.00$
O	21	$0.12^{cd} \pm 0.00$	$0.12^{cde} \pm 0.00$	$0.03^{cd} \pm 0.01$	$0.25^{cde} \pm 0.00$
O	22	$0.12^{cd} \pm 0.00$	$0.08^{cde} \pm 0.02$	$0.07^{cd} \pm 0.03$	$0.09^{de} \pm 0.01$
O	24	$0.12^{cd} \pm 0.00$	$0.12^{cde} \pm 0.00$	$0.03^{cd} \pm 0.01$	$0.28^{bcde} \pm 0.12$
O	25	$0.10^{cd} \pm 0.01$	$0.12^{cde} \pm 0.00$	$0.10^{cd} \pm 0.05$	$0.09^{de} \pm 0.01$
O	26	$0.21^{bcd} \pm 0.03$	$0.18^{bcde} \pm 0.03$	$0.15^{bcd} \pm 0.05$	$0.18^{cde} \pm 0.03$
O	28	$0.50^{abcd} \pm 0.00$	$0.07^{de} \pm 0.02$	$0.04^{cd} \pm 0.009$	$0.25^{cde} \pm 0.00$
O	29	$0.18^{bcd} \pm 0.03$	$0.06^{de} \pm 0.00$	$0.14^{bcd} \pm 0.06$	$0.18^{cde} \pm 0.03$
O	31	$0.62^{abcd} \pm 0.21$	$0.13^{cde} \pm 0.06$	$0.05^{cd} \pm 0.007$	$0.12^{de} \pm 0.00$
O	32	$0.75^{ab} \pm 0.14$	$0.10^{cde} \pm 0.05$	$0.13^{bcd} \pm 0.06$	$0.12^{de} \pm 0.00$
O	33	$0.50^{abcd} \pm 0.00$	$0.09^{cde} \pm 0.01$	$0.07^{cd} \pm 0.03$	$0.10^{de} \pm 0.01$
O	34	$0.75^{ab} \pm 0.14$	$0.25^{bcde} \pm 0.00$	$0.04^{cd} \pm 0.01$	$0.12^{de} \pm 0.00$
O	35	$0.53^{abcd} \pm 0.27$	$0.04^{e} \pm 0.09$	$0.03^{cd} \pm 0.01$	$0.12^{de} \pm 0.00$
O	36	$0.25^{bcd} \pm 0.08$	$0.12^{cde} \pm 0.00$	$0.08^{cd} \pm 0.02$	$0.18^{cde} \pm 0.03$
O	41	$0.75^{ab} \pm 0.14$	$0.13^{cde} \pm 0.06$	$0.09^{cd} \pm 0.01$	$0.12^{de} \pm 0.00$
O	47	$0.28^{bcd} \pm 0.07$	$0.37^{bcd} \pm 0.07$	$0.18^{bcd} \pm 0.03$	$0.14^{de} \pm 0.03$
B	3	$0.18^{bcd} \pm 0.03$	$0.03^{e} \pm 0.00$	$0.02^{cd} \pm 0.004$	$0.18^{cde} \pm 0.03$
B	5	$0.31^{bcd} \pm 0.06$	$0.37^{bcd} \pm 0.07$	$0.17^{bcd} \pm 0.04$	$0.50^{b} \pm 0.00$
B	6	$0.21^{bcd} \pm 0.03$	$0.14^{cde} \pm 0.06$	$0.03^{cd} \pm 0.00$	$0.37^{bc} \pm 0.07$
B	13	$0.31^{bcd} \pm 0.06$	$0.18^{bcde} \pm 0.03$	$0.28^{bcd} \pm 0.12$	$0.18^{cde} \pm 0.03$
B	15	$0.18^{bcd} \pm 0.03$	$0.50^{ab} \pm 0.00$	$0.13^{bcd} \pm 0.06$	$0.12^{de} \pm 0.00$
B	20	$0.12^{cd} \pm 0.00$	$0.15^{cde} \pm 0.03$	$0.08^{cd} \pm 0.02$	$0.50^{b} \pm 0.00$
B	23	$0.21^{bcd} \pm 0.03$	$0.37^{bcd} \pm 0.07$	$0.10^{bcd} \pm 0.05$	$0.18^{cde} \pm 0.03$
B	37	$0.62^{abcd} \pm 0.21$	$0.28^{bcde} \pm 0.12$	$0.31^{bc} \pm 0.10$	$0.50^{b} \pm 0.00$
B	38	$0.62^{abcd} \pm 0.21$	$0.15^{cde} \pm 0.05$	$0.07^{cd} \pm 0.02$	$0.25^{cde} \pm 0.00$
B	39	$0.56^{abcd} \pm 0.25$	$0.18^{bcde} \pm 0.03$	$0.14^{bcd} \pm 0.05$	$0.37^{bc} \pm 0.07$
B	40	$0.68^{abc} \pm 0.18$	$0.50^{ab} \pm 0.00$	$1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$
B	43	$0.68^{abc} \pm 0.18$	$0.14^{cde} \pm 0.06$	$0.32^{bc} \pm 0.10$	$0.50^{b} \pm 0.00$
B	45	$> 1.00^{a} \pm 0.00$	$0.75^{a} \pm 0.14$	$0.25^{bcd} \pm 0.00$	$> 1.00^{a} \pm 0.00$
B	48	$0.37^{bcd} \pm 0.07$	$0.17^{cde} \pm 0.04$	$0.07^{cd} \pm 0.02$	$0.31^{bcd} \pm 0.10$
M	30	$0.25^{bcd} \pm 0.00$	$0.37^{bcd} \pm 0.07$	$0.14^{bcd} \pm 0.06$	$1.00^{a} \pm 0.00$
Antibiotics	**Rif**	$0.20^{bcd} \pm 0.00$	$0.10^{cde} \pm 0.00$	$0.006^{d} \pm 0.00$	NT
	Stp	$0.02^{d} \pm 0.00$	$0.02^{e} \pm 0.00$	$> 0.40^{b} \pm 0.00$	NT
	Amp	$> 0.40^{abcd} \pm 0.00$	$> 0.40^{bc} \pm 0.00$	$> 0.40^{b} \pm 0.00$	NT
	Nys	NT	NT	NT	$0.40^{bc} \pm 0.00$

*Values followed by the same letter in the each column and isolate, are not significantly different ($P < 0.05$), according to Tukey's HSD test.

O – Orange subtype of propolis, B – Blue subtype of propolis, M – Third subtype of propolis

Rif - Rifampicin, Stp - Streptomycin, Amp - Ampicillin, Nys – Nystatin, NT – Not tested.

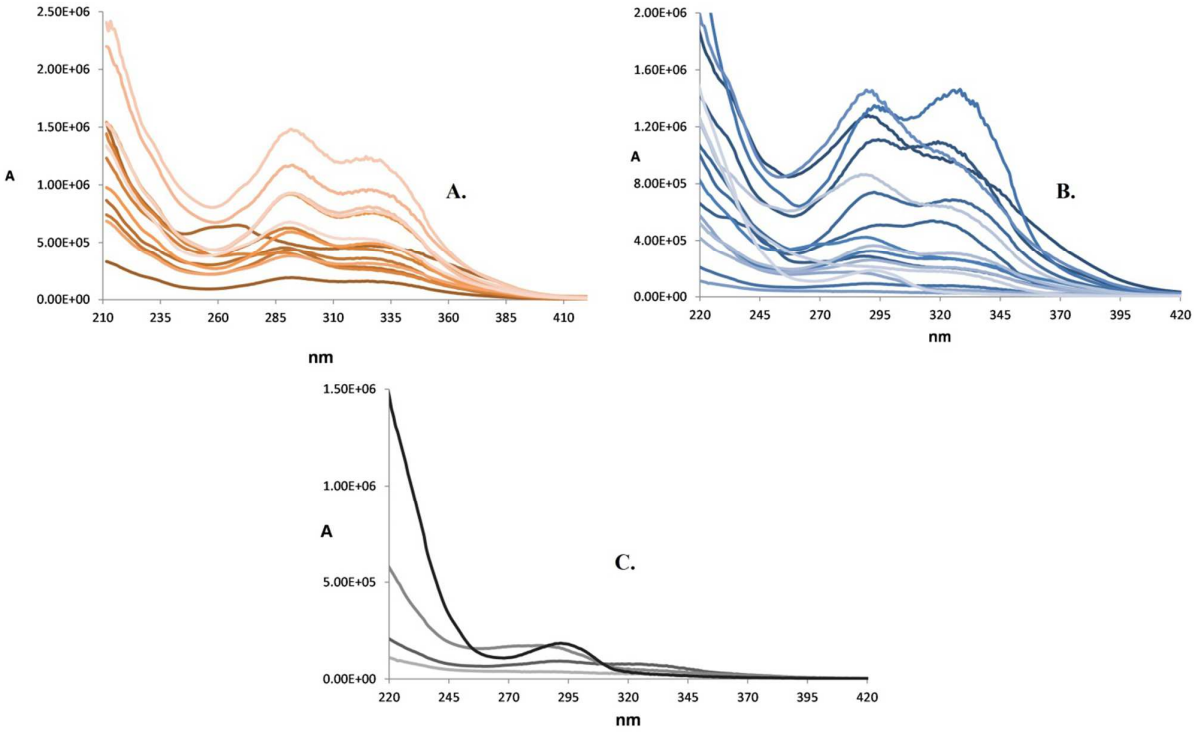
Table 4. The minimum bactericidal (MBC) and fungicidal concentrations (MFC) of Turkish propolis samples (mg/mL). The mean values and standard error are shown.

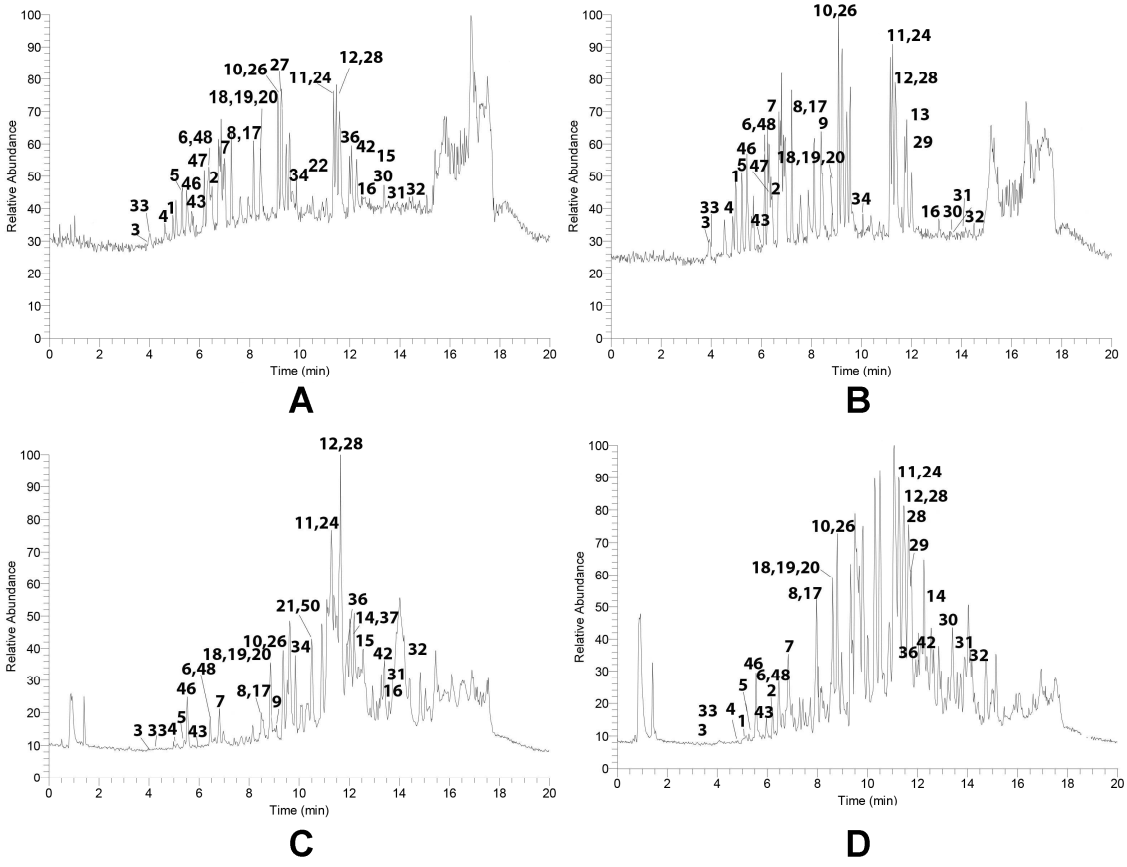
Subtype of propolis	Sample	MBC			MFC
		S. sanguinis	***S. mutans***	***S. pyogenes***	***C. albicans***
O	2	$1.00^{a} \pm 0.00$	$0.56^{abc} \pm 0.25$	$0.28^{bc} \pm 0.12$	$> 1.00^{a} \pm 0.00$
O	4	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$1.00^{a} \pm 0.00$
O	7	$0.37^{bc} \pm 0.07$	$0.75^{ab} \pm 0.14$	$0.50^{abc} \pm 0.00$	$1.00^{a} \pm 0.00$
O	8	$0.37^{bc} \pm 0.07$	$0.18^{bc} \pm 0.03$	$0.75^{ab} \pm 0.14$	$0.50^{e} \pm 0.00$
O	11	$0.37^{bc} \pm 0.07$	$0.56^{abc} \pm 0.25$	$0.37^{bc} \pm 0.07$	$0.18^{de} \pm 0.03$
O	12	$0.25^{bc} \pm 0.00$	$0.37^{bc} \pm 0.07$	$0.31^{bc} \pm 0.10$	$0.50^{bcde} \pm 0.00$
O	16	$0.50^{abc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$1.00^{a} \pm 0.00$	$0.62^{abcd} \pm 0.21$
O	17	$0.37^{bc} \pm 0.07$	$0.75^{ab} \pm 0.14$	$0.37^{bc} \pm 0.07$	$1.00^{a} \pm 0.00$
O	18	$0.56^{abc} \pm 0.15$	$0.18^{bc} \pm 0.03$	$0.31^{bc} \pm 0.10$	$0.12^{bcde} \pm 0.00$
O	21	$0.25^{bc} \pm 0.00$	$0.25^{bc} \pm 0.00$	$0.31^{bc} \pm 0.10$	$0.75^{ab} \pm 0.14$
O	22	$0.25^{bc} \pm 0.00$	$0.18^{bc} \pm 0.03$	$0.18^{bc} \pm 0.03$	$0.18^{de} \pm 0.03$
O	24	$0.37^{bc} \pm 0.07$	$0.25^{bc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.56^{abcde} \pm 0.25$
O	25	$0.37^{bc} \pm 0.07$	$0.25^{bc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.18^{de} \pm 0.03$
O	26	$0.50^{abc} \pm 0.00$	$0.50^{abc} \pm 0.00$	$0.37^{bc} \pm 0.07$	$0.37^{bcde} \pm 0.07$
O	28	$1.00^{a} \pm 0.00$	$0.25^{bc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.50^{bcde} \pm 0.00$
O	29	$0.62^{ab} \pm 0.21$	$0.37^{bc} \pm 0.07$	$0.50^{abc} \pm 0.00$	$0.37^{bcde} \pm 0.07$
O	31	$1.00^{a} \pm 0.00$	$1.00^{a} \pm 0.00$	$0.56^{abc} \pm 0.25$	$0.25^{cde} \pm 0.00$
O	32	$1.00^{a \pm} 0.00$	$0.53^{abc} \pm 0.27$	$0.37^{bc} \pm 0.07$	$0.25^{cde} \pm 0.00$
O	33	$1.00^{a} \pm 0.00$	$0.37^{bc} \pm 0.07$	$0.18^{bc} \pm 0.03$	$0.25^{cde} \pm 0.00$
O	34	$1.00^{a} \pm 0.00$	$0.50^{abc} \pm 0.00$	$0.50^{abc} \pm 0.00$	$0.37^{bcde} \pm 0.07$
O	35	$0.62^{ab} \pm 0.21$	$0.15^{bc} \pm 0.05$	$0.18^{bc} \pm 0.03$	$0.37^{bcde} \pm 0.07$
O	36	$0.62^{ab} \pm 0.21$	$0.37^{bc} \pm 0.07$	$0.37^{bc} \pm 0.07$	$0.37^{bcde} \pm 0.07$
O	41	$> 1.00^{a} \pm 0.00$	$0.28^{bc} \pm 0.12$	$0.50^{abc} \pm 0.00$	$0.25^{cde} \pm 0.00$
O	47	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.68^{abc} \pm 0.18$
B	3	$0.50^{abc} \pm 0.00$	$0.06^{c} \pm 0.00$	$0.28^{bc} \pm 0.12$	$0.37^{bcde} \pm 0.07$
B	5	$1.00^{a} \pm 0.00$	$1.00^{a} \pm 0.00$	$0.50^{abc} \pm 0.00$	$1.00^{a} \pm 0.00$
B	6	$0.75^{ab} \pm 0.14$	$0.37^{bc} \pm 0.07$	$0.18^{bc} \pm 0.03$	$0.75^{ab} \pm 0.14$
B	13	$1.00^{a} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.37^{bcde} \pm 0.07$
B	15	$0.37^{bc} \pm 0.07$	$1.00^{a} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.25^{cde} \pm 0.00$
B	20	$0.25^{bc} \pm 0.00$	$0.37^{bc} \pm 0.07$	$0.25^{bc} \pm 0.00$	$1.00^{a} \pm 0.00$
B	23	$0.50^{abc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.37^{bcde} \pm 0.07$
B	37	$1.00^{a} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$1.00^{a} \pm 0.00$
B	38	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.50^{bcde} \pm 0.00$
B	39	$0.75^{ab} \pm 0.14$	$0.75^{ab} \pm 0.14$	$0.50^{abc} \pm 0.00$	$0.75^{ab} \pm 0.14$
B	40	$> 1.00^{a} \pm 0.00$	$1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$
B	43	$> 1.00^{a} \pm 0.00$	$0.37^{bc} \pm 0.07$	$> 1.00^{a} \pm 0.00$	$1.00^{a} \pm 0.00$
B	45	$> 1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$	$> 1.00^{a} \pm 0.00$
B	48	$0.75^{ab} \pm 0.14$	$0.50^{abc} \pm 0.00$	$0.75^{ab} \pm 0.14$	$0.62^{abcd} \pm 0.21$
M	30	$0.75^{ab} \pm 0.14$	$1.00^{a} \pm 0.00$	$0.50^{abc} \pm 0.00$	$> 1.00^{a} \pm 0.00$
Antibiotics	**Rif**	$0.40^{bc} \pm 0.00$	$0.40^{abc} \pm 0.00$	$0.10^{c} \pm 0.00$	NT
	Stp	$0.05^{c} \pm 0.00$	$0.05^{c} \pm 0.00$	$> 0.40^{bc} \pm 0.00$	NT
	Amp	$> 0.40^{bc} \pm 0.00$	$> 0.40^{abc} \pm 0.00$	$> 0.40^{bc} \pm 0.00$	NT
	Nys	NT	NT	NT	$> 0.40^{bcde} \pm 0.00$

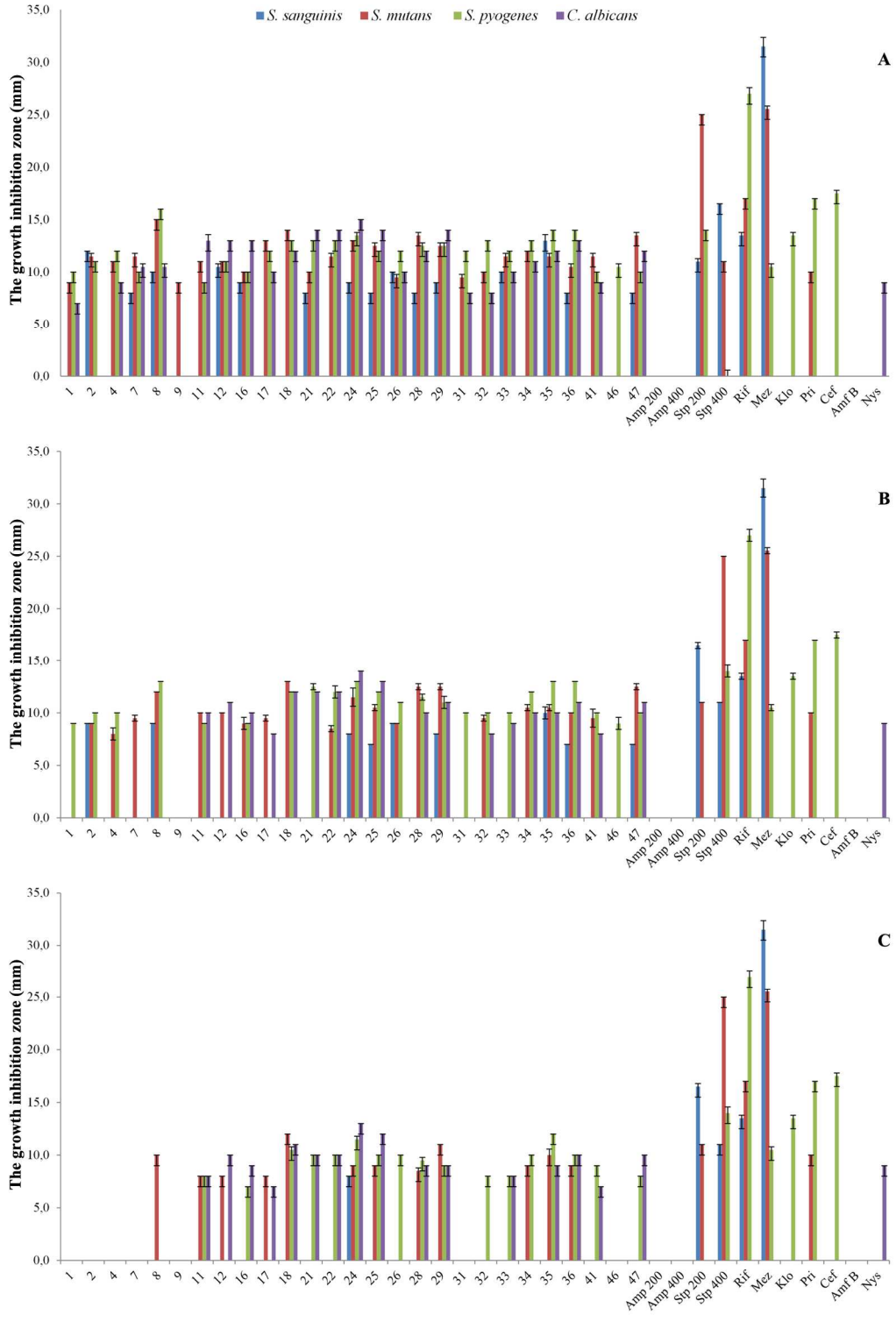
*Values followed by the same letter in the each column and isolate, are not significantly different ($P < 0.05$), according to Tukey's HSD test.

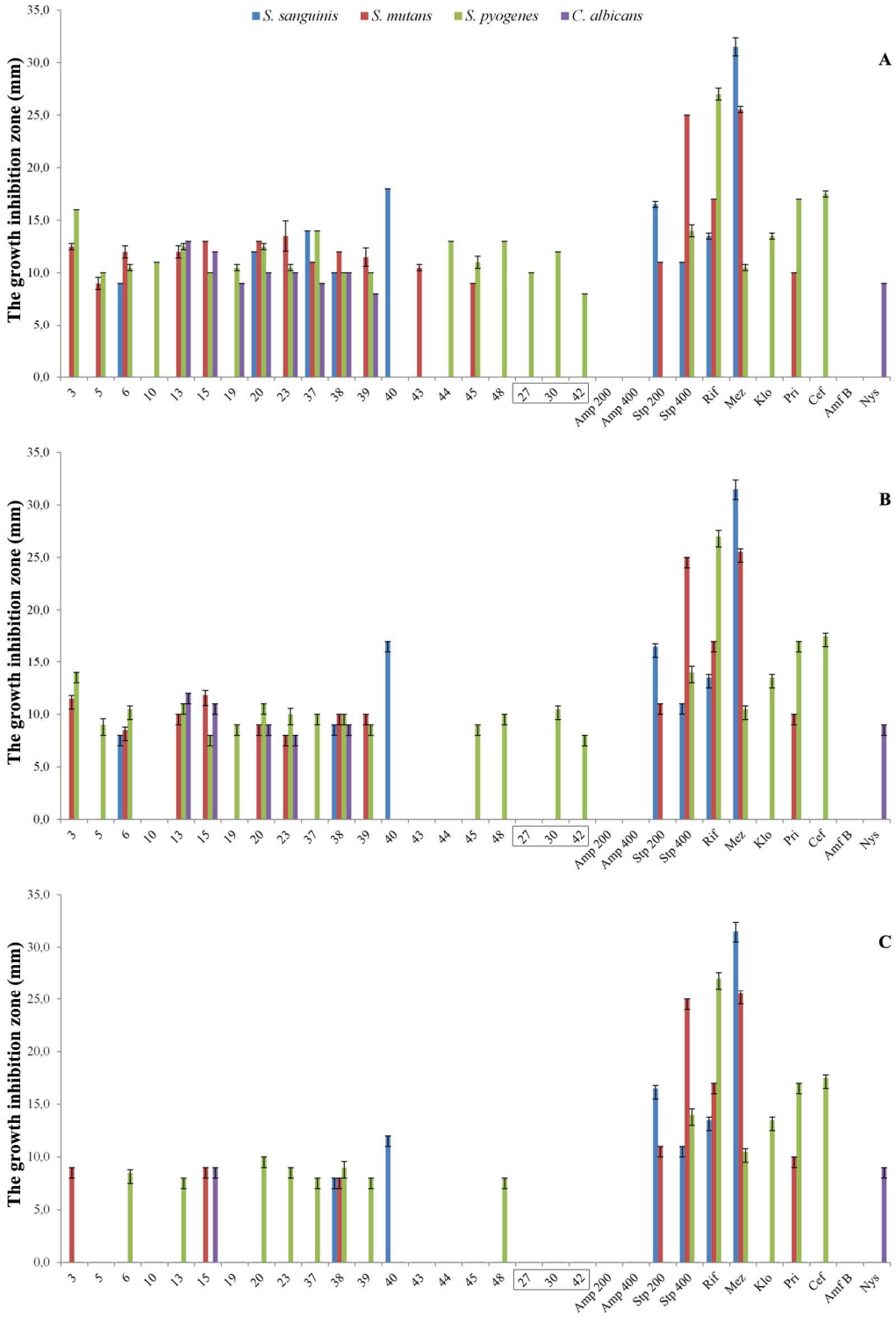
O – Orange subtype of propolis, B – Blue subtype of propolis, M – Third subtype of propolis

Rif - Rifampicin, Stp - Streptomycin, Amp - Ampicillin, Nys – Nystatin, NT – Not tested.






S. sanguinis
S. mutans
S. pyogenes
C. albicans
A
B
C
The growth inhibition zone (mm)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
1 2 4 7 8 9 11 12 16 17 18 21 22 24 25 26 28 29 31 32 33 34 35 36 41 46 47 Amp 200 Amp 400 Stp 200 Stp 400 Rif Mez Klo Pri Cef AmfB Nys


S. sanguinis
S. mutans
S. pyogenes
C. albicans
The growth inhibition zone (mm)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
A
B
C
3
5
6
10
13
15
19
20
23
37
38
39
40
43
44
45
48
27
30
42
Amp 200
Amp 400
Stp 200
Stp 400
Rif
Mez
Klo
Pri
Cef
AmfB
Nys

Highlights

- Phenolic profiling of three subtypes of Turkish poplar type propolis was studied.
- Quality control parameters of three subtypes of propolis were investigated.
- O-subtype propolis had higher total phenolic and flavonoid contents than B- subtype.
- O- subtype of propolis showed higher antioxidative and antimicrobial activities.