Accepted Manuscript

Authentication of Turkish propolis through HPTLC fingerprints combined with multivariate analysis and palynological data and their comparative antioxidant activity

Etil Guzelmeric, Petar Ristivojević, Jelena Trifković, Tugce Dastan, Ozlem Yilmaz, Ozlem Cengiz, Erdem Yesilada

PII: S0023-6438(17)30630-8

DOI: 10.1016/j.lwt.2017.08.060

Reference: YFSTL 6482

To appear in: *LWT - Food Science and Technology*

Received Date: 8 April 2017

Revised Date: 11 July 2017

Accepted Date: 19 August 2017

Please cite this article as: Guzelmeric, E., Ristivojević, P., Trifković, J., Dastan, T., Yilmaz, O., Cengiz, O., Yesilada, E., Authentication of Turkish propolis through HPTLC fingerprints combined with multivariate analysis and palynological data and their comparative antioxidant activity, *LWT - Food Science and Technology* (2017), doi: 10.1016/j.lwt.2017.08.060.

Authentication of Turkish propolis through HPTLC fingerprints combined with multivariate analysis and palynological data and their comparative antioxidant activity

Etil Guzelmeric[a*], Petar Ristivojević[b,c], Jelena Trifković[d], Tugce Dastan[e], Ozlem Yilmaz[e], Ozlem Cengiz[e], Erdem Yesilada[a]

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

[b]Innovation Centre of Faculty of Chemistry Ltd, Studentski trg 12-16, 11000 Belgrade, Serbia

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

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

[e]Balparmak Research Center, Altiparmak Gida San. Tic. A. S., Cavusbasi Cad., Cekmekoy 34782, Istanbul, Turkey

***Corresponding author:** Assist. Prof. Dr. Etil Guzelmeric

Tel: +90 216 578 00 00

E-mail address: etil.ariburnu@yeditepe.edu.tr

1. Introduction

Propolis is a complex resinous product prepared by honeybees (*Apis meliffera* L.) from buds, sprouts and exudates of various plants after subjecting some enzymatic changes. It helps to repair the cracks and crevices of the honeycomb, to protect beehive against microbial contamination and to preserve inner temperature (Salatino, Teixeira, Negri, & Message, 2005).

Chemical composition of propolis exerts high variability depending upon the geographical and climate factors as well as vegetation around the beehives. The specificity of local flora determines propolis affiliation to a particular group. Plant buds' resin such as *Poplar* sp. (*Populus alba*, *Populus tremula* and *Populus nigra*) have been reported as a primary source of propolis in temperate zones (Europe, North America, and non-tropical regions of Asia). Secondarily important sources of European propolis are reported to be *Betula pendula, Acacia* sp, *Aesculus hippocastanum*, *Alnus glutinosa*, *Pinus* sp. and *Salix alba* (Kumova, Korkmaz, Avcı, & Ceyran, 2002; Ristivojević, Trifković, Andrić, & Milojković-Opsenica, 2015; Yesilada, 2015).

Each propolis type is characterized by the specific proportion of the dominant plant material. Various chromatographic techniques were used for evaluation of the botanical origin of propolis samples such as high performance thin-layer chromatography (HPTLC) (Bertrams, Müller, Kunz, Kammerer, & Stintzing, 2013; Morlock, Ristivojevic, & Chernetsova, 2014; Ristivojević et al., 2014; Sârbu & Moţ, 2011), high-performance liquid chromatography (HPLC) (Falcão et al., 2013; Park, Alencar, & Aguiar, 2002), gas chromatography-mass spectrometry (GC-MS) (Greenaway, Scaysbrook, & Whatley, 1987), capillary electrophoresis (CE) (Cao, Wanh, & Yuan, 2004), microscopic techniques (Barth, 1998; Barth & Fernandes

Pinto da Luz, 2009; Moreira, Dias, Pereira, & Estevinho, 2008; Warakomska & Maciejewicz, 1992). Furthermore, structure elucidation techniques such as direct analysis in real time (DART) (Morlock et al., 2014), nuclear magnetic resonance (NMR) (Bertelli, Papotti, Bortolotti, Marcazzan, & Plessi, 2012), and infrared spectroscopy (IR) (Nie, Xia, Sun, & He, 2013) have been applied in order to monitor compositional differences between propolis samples supplied from various origins.

Propolis has been utilized for treatment of a wide range of diseases in traditional medicines dating back to 300 BC (Ghisalberti, 1979). Recent studies have also evidenced its healing benefits such as anticancer, antioxidant, anti-inflammatory, antimicrobial, antiulcer, antidiabetic and antihepatotoxic effects (Huang, Zhang, Wang, Li, & Hu, 2014; Yesilada, 2015).

Since function of propolis in all hives are same, despite of different chemical composition, biological properties of different propolis types are almost similar, i.e. propolis always possesses considerable biological activity, but in different potency. In that sense, biological activity of propolis should always be reported together with its chemical characterization (Ristivojević et al., 2015). Prediction of biological activity of a propolis sample based on the quantification of its individual components may not be rational due to the fact that each compound may possess different activity profiles (Bankova, 2005) and possible synergistic interactions between the components in the mixture (Boisard et al., 2014). Namely, determination of specific chemical profile in order to mark a group of active compounds would be a better solution for assessment of a particular biological activity of propolis. Biological activity of propolis is mainly attributed to its phenolic constituents, *i.e.* flavonoids (including flavones, flavonols, flavanones and dihydroflavonols) and other phenolics (mainly

substituted cinnamic acids and their esters) (Huang et al., 2014) and one of the well-known biological activities of phenolics is their antioxidant effect.

HPTLC coupled with 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) detection is a handy technique for screening of antioxidant capacity of each separated component in a mixture or extract (Cieśla, Kryszeń, Stochmal, Oleszek, & Waksmundzka-Hajnos, 2012). Determination of total antioxidant activity of propolis using *in vitro* DPPH˙ assay was reported by many researches (Isla, Paredes-Guzman, Nieva-Moreno, Koo, & Park, 2005; Shi, Yang, Zhang, & Yu, 2012). However, there are only few studies for evaluation of antioxidant components in propolis by application of HPTLC-DPPH˙(Bertrams et al., 2013).

Turkey is located in between Europe and Asia, surrounded by seas (the Marmara Sea, Aegean Sea, Black Sea and Mediterranean Sea) with substantially different climate zones. For this reason, from the viewpoint of plant diversity, the Turkish flora consists of more than 11.000 infrageneric taxa, approximately one third of them being endemic (Baser, 2002). However, only a few investigations related to chemical composition and pharmacological activities of Turkish propolis have been carried out so far. In these studies only the GC-MS profiles of the Turkish propolis samples were investigated (Sorkun, Süer, & Salih, 2001; Popova, Silici, Kaftanoglu, & Bankova, 2005; Duran, Muz, Culha, Duran, & Ozer, 2011).

HPTLC profiling is particularly applied for natural extracts as a simple, rapid, and low-cost technique for determination of authenticity according to a set of characteristic chromatographic signals, which comparison leads to sample recognition.

Based on the facts discussed above, the aims of this study were assessed as follows: 1) investigation of HPTLC phenolic profile of Turkish propolis samples 2) determination of botanical origin of Turkish propolis samples by simultaneous profiling of different bud

extracts as potential botanical sources, 3) investigation of possible application of HPTLC fingerprint in combination with multivariate image analysis and pattern recognition technique for the assessment of botanical and geographical origin of Turkish propolis samples, 4) determination of botanical origin of Turkish propolis samples by characterization of pollen grains in samples, 5) evaluation of both total antioxidant capacity (total peak area of the separated compounds on the chromatogram) and contribution of each antioxidant components, in Turkish propolis samples, using HPTLC-DPPH˙ assay.

2. Experimental

2.1. Chemicals and solvents

Ethyl acetate, ethanol, toluene, *n*-hexane and acetic anhydride were purchased from Sigma-Aldrich (Steinheim, Germany); acetic acid was from Riedel-de Haen (Seelze, Germany). 2-aminoethyl diphenylborinate was obtained from Fluka (Steinheim, Germany). Polyethylene glycol 400, glycerol, gelatine, carbol fuchsin, phenol, sulphuric acid and potassium hydroxide (KOH) were purchased from Merck (Hohenbrunn, Germany), respectively. All solvents were analytical purity grade.

Standards of pinocembrin, galangin, pinobanksin, caffeic acid phenyl ester (CAPE), naringenin, caffeic acid, chrysin, quercetin and apigenin were purchased from Sigma-Aldrich (Steinheim, Germany). List of standard compounds with hR_F values and color of bands are presented in Table 1.

2.2. Propolis samples

Sixty crude propolis samples collected by professional beekeepers were from different regions of Turkey (48 propolis samples, encoded as P1-48) and Serbia (12 propolis samples, encoded as P49-60). The localities of samples from Turkey were shown in Figure 1. Moreover, list of

all samples with their geographical origins, classification according to TLC fingerprinting, and collection dates were presented in Supplemental Table S1.

2.3. Plant bud samples

The buds belong to *Populus nigra* L., *Populus tremula* L., *Populus alba* L., *Betula pendula* L., *Quercus petraea* Matt., *Salix caprea* L., *Ulmus glabra* L., *Tilia tomentosa* L., *Tilia americana* L., *Tilia* x *europaea 'Pallida'* were collected from trees in campus area at Yeditepe University (Istanbul, Turkey) and Ataturk Arboretum (Istanbul, Turkey). Moreover, one sample belong to *P. nigra* bud was gathered from mountain area Fruška gora (Serbia).

2.4. Preparation of standard solutions

Pinocembrin, galangin, CAPE, naringenin, caffeic acid, chrysin, quercetin, apigenin stock solutions (0.1 mg/mL) were prepared in methanol and mixed to prepare standard mixture solution (STD MIX) in volume ratios of 1:1:1:0.5:1:0.5:0.5:0.5:0.5, respectively.

2.5. Preparation of detection reagents

Natural Products (NP) dipping solution was prepared by dissolving 1 g of 2-aminoethyl diphenylborinate in 200 mL of ethyl acetate. Polyethylene glycol (PEG) 400 dipping solution was prepared by dissolving 10 g of polyethylene glycol 400 in 200 mL of dichloromethane (Reich & Schibli, 2007).

Anisaldehyde reagent was prepared by adding 10 mL of sulphıric acid to an iced-cooled mixture of 170 mL methanol and 20 mL of acetic acid. Then, 1 mL anisaldehyde was added to this solution (Reich & Schibli, 2007).

2.6. Preparation of glycerine jelly

Seven grams of gelatine was mixed with cold distilled water and then warmed gently and stirred until complete dissolution. Then, 50 mL glycerol and 0.5 g phenol were added. For

staining, carbol fuchsin was added drop by drop to obtain a clear pink solution. It was poured into petri dishes and then let to solidify (Sawyer, 1988).

2.7. Preparation of acetolysis mixture

Acetic anhydride and concentrated sulphuric acid was mixed in volume ratios of 9:1 (Barth, 1998).

2.8. Preparation of sample test solutions

2.8.1. Propolis extraction for screening phenolic compounds

Accurately weighted 1 g of each raw propolis samples was cut into small pieces and then extracted with 10 mL ethanol-water [8:2 volume ratio] in an ultrasonic bath for 45 min. Then the extract was centrifuged at 5300 rpm for 30 min and upper phase was evaporated to dryness with rotary evaporator and the residue was then dissolved in 5 mL of ethanol. Each ethanolic solution was filtered through a 0.45 µm RC-membrane filter (Sartorius stedim biotech) and was diluted 15 times and kept refrigerated at -20ºC prior to analysis.

2.8.2. Propolis extraction for screening terpenic compounds

One gram of each raw propolis sample was accurately weighed and comminuted. Then each sample was extracted with 10 mL chloroform in an ultrasonic bath for 45 minutes. After filtration, the solution was evaporated to dryness under reduced pressure. Finally, the residue was dissolved in 5 mL of chloroform and then diluted 5 times with the same solvent.

2.8.3. Propolis extraction for palynological analysis

Palynological processing of the propolis samples performed according the standard methodology (Barth, 1998; Barth & Fernandes Pinto da Luz, 2009), using 0.5 g of scraped propolis. Samples were extracted overnight with ethanol by continuous stirring. After the mixture was centrifuged at 4000 rpm for 10 min, the sediment was treated with 10%

potassium hydroxide solution and heated for 2 min in a water bath. Each tube was put in an ultrasonic mixer for 5 min and then sieved through 0.3 mm mesh sieve to remove large organic particles. After application of acetolysis process to the filtrate, the sediment obtained was washed with 5 mL distilled water and centrifuged at 4000 rpm for 5 min (Erdtman, 1952). The supernatant was discarded after centrifugation. The sediment was resuspended with 0.2 mL distilled water and spread onto a microscope slide. The microscope slides were put onto the heating plate and stained with glycerine jelly containing carbol fuchsin.

2.8.4. Plant bud extraction

One gram of each bud sample was cut into small pieces and extracted with 20 mL of ethanol at 70°C in a water-bath with continuous shaking. After filtration, ethanol was removed using a rotary evaporator and the residue was dissolved in 5 mL of ethanol.

2.9. Microscopic examination

Pollen grains were examined using Nikon Eclipse 80i digital microscopy (Clemex Vision PE: image analysis) and determined by 40x and immersion objectives (100x). For the identification, type, shape and size of the pollen grain, thickness and ornamentation type of exine, aperture number, place of the apertures on pollen grain, size and type of aperture as well as pore and cracks properties of pollens were examined. Results were evaluated according to the following scaling system: Dominant pollen (DP) (>45%); secondary pollen (SP) (15 to 45%); important minor pollen (IMP) (3 to 15%) and minor pollen (MP) (<3%) (Barth, 1998).

2.10. HPTLC method

Each sample test solutions of hydroalcoholic propolis extracts (5 µL), propolis chloroform extracts (2 µL), plant bud extracts (2 µL) and also standard mixture solution (40µL) were

184 applied on HPTLC glass plates (20 x 10 cm) precoated with silica gel 60 F_{254} (Merck) using a
185 Linomat V automatic sample spotter (Camag, Muttenz, Switzerland) equipped with 100 µL
186 Hamilton syringe (Bonaduz, Switzerland).
187 For the chromatographic separation of phenolic compounds, HPTLC method reported by
188 Morlock et al. (Morlock et al., 2014) was modified in order to optimize the resolution of the
189 phenolic acids and flavonoids. Optimization included: 1) different proportions of hydrochloric
190 acid were used for preconditioning (32-37%), 2) different acids (acetic acid and formic acid)
191 were tested for preconditioning, 3) different amounts of hydrochloric acid (5-10 mL) were
192 used to wet filter paper, 4) the time necessary for preconditioning (5-20 min) was adjusted, 5)
193 Conditioning of the chamber was performed with hydrochloric acid 37% without using a filter
194 paper.
195 In this study, 10 mL of mobile phase composed of *n*-hexane-ethyl acetate-glacial acetic acid
196 in volume ratios of 5:3:1 was placed in one trough of the twin-trough chamber (20 x 10 cm)
197 (Camag), while 10 mL of 37% hydrochloric acid was put in the other trough. The chamber
198 was saturated for 20 min. Then, the plate was developed up to a migration distance of 65 mm.
199 After development, the plate was dried under a warm air flow for 5 min.
200 For terpenic compounds, the plate was developed with the solvent system of toluene-ethyl
201 acetate [95:5 volume ratio] up to 7 cm in a saturated (20 min) twin-trough chamber (Reich &
202 Schibli, 2007). After development, the plate was dried under a warm air flow for 5 min.
203 Anisaldehyde, NP and PEG derivatization reagents were prepared according to section 2.5.
204 For derivatization of phenolic compounds, the plates were first heated at 100ºC on the Camag
205 TLC plate heater for 3 min and dipped into NP and PEG 400 solutions, respectively. To
206 screen terpenic compounds, the plate was dipped into anisaldehyde solution and afterwards

heated on 100˚C for 3 min. After derivatization, the plates were documented by the TLC visualizer (Camag) at 366 nm for phenolic compounds, and at 366 nm and under white light in reflectance mode for terpens. All the instruments were operated by winCATS program (Version 1.4.8.2031, Camag).

2.11. HPTLC-DPPH˙ test

HPTLC-DPPH˙ assay was used to detect the active antioxidative constituents separated on the plate. Applied chromatographic conditions were those described above. After drying, the plate was dipped into freshly prepared 0.1% methanolic DPPH˙solution. Immediately after, image capturing was performed under white light in reflectance mode, by TLC visualizer. Finally, the images were evaluated by Camag VideoScan TLC evaluation software (version 1.02.00).

2.12. Image analysis and multivariate analysis

Image analysis was applied as the first step to convert HPTLC chromatograms to numerical data sets. Images of the chromatograms were processed with the Image J program (http://imagej.nih.gov/ij/, ver. 1.47q, Rasband W. National Institutes of Health, USA) as it was described by Ristivojević et al. (Ristivojević et al., 2014). Before image analysis, each chromatogram was divided into three channels (red, green and blue). Denoising of the images was achieved using 2 pixels median filter. Differences of the background intensity between images were removed with the use of a bandpass filter (filter large structures down to 40 pixels; filter small structures up to 3 pixels). Normalization of the images was performed by using Standard Normal Variate (SNV) transformation which removes the slope variation from chromatogram caused by scattering and variation of the particle size (Candolfi, De Maesschalck, Jouan-Rimbaud, Hailey, & Massart, 1999). The warping of the images was done with correlation optimized warping (COW) algorithm implemented in the PLS Tool

Box, v.6.2.1, for MATLAB (7.12.0 (R2011a) ("Eigenvector Research., Inc," n.d.), using auto selection of target sample.

Principal component analysis (PCA) was applied on the matrix (48 samples × 427 variables) obtained by digitization of chromatograms of phenolic compounds documented at 366 nm, for each channel separately. Variables represent the intensities of pixels along the 427 length lines. The data were additionally processed before data analysis by using mean centering, which is the preferred option when the classification of samples is based on variables that are all measured in the same unit. The number of principal components (PCs) was determined according to scree plot, which represents the ability of PCs to explain the variation in the data. PCA was performed with PLS Tool Box v.6.2.1, for MATLAB 7.12.0 (R2011a) (http://www.eigenvector.com/software/pls_toolbox.htm, Eigenvector Research, Inc., Wenatchee, WA 98801). It was carried out as an exploratory data analysis by using a singular value decomposition algorithm (SVD) and a 0.95 confidence level for Q and T^2 Hotelling limits for outliers.

3. Results and discussion

In the previous reports, based on the band colors of phenolic profiles in HPTLC analysis of propolis samples originating from Serbia, Slovenia, Croatia, Germany and Romania, the authors have suggested the presence of two main varieties of European poplar propolis, orange (O) and blue (B) types (Bertrams et al., 2013; Milojković-Opsenica et al., 2016; Morlock et al., 2014; Sârbu & Moţ, 2011; Ristivojević et al., 2014). O-type propolis shows presence of several strong orange colored bands together with small number of light blue and faint green bands, while B-type is characterized with deep and light blue bands and weak orange and green bands. Strong orange bands are typical for flavonoids like quercetin, blue

bands for caffeic acid, CAPE, galangin, feruloyl and *p*-coumaroyl derivatives, and green ones correspond to apigenin, apigenin-methyl-ether or naringenin (Bertrams et al., 2013; Morlock et al., 2014; Ristivojević et al., 2014). Extract of the black poplar (*Populus nigra* L.) buds showed a very similar pattern to the O-type samples which is most likely to represent the origin of this type. The B-type was found to be correlated to a certain extent with the aspen (*Populus tremula*). In addition, horse chestnut (*Aesculus hippocastanum* L.) components were detected in both types (Morlock, Scholl, Kunz, & Schroeder, 2013).

3.1. HPTLC phenolic profile of Turkish propolis

Complete chemical characterization of propolis, as a complex natural matrix with highly variable chemical composition, is difficult to perform. TLC-fingerprinting is usually a useful technique, emphasizing a set of characteristic chromatographic signals, which would enable sample recognition. In that sense, in the present investigation, the HPTLC fingerprint was performed for initial screening of Turkish propolis in order to verify botanical and geographical differences between the investigated samples. The analysis was performed together with samples from Serbia as a typical representative of European poplar type propolis (Supplemental Figure S1) (Ristivojević et al., 2014). A mixture of nine standard solutions was also analyzed simultaneously with Turkish and Serbian propolis samples.

Visual comparison of the HPTLC chromatograms of Turkish propolis samples have let the detection of an additional type of chemical pattern other than O- or B-types of European propolis. This new type of Turkish propolis was mainly composed of nonphenolic compounds (Supplemental Table S1), having an unusual chemical profile without characteristics bands for phenolics and was introduced for the first time in this study. Concerning the diversity of the Turkish flora that consists of high number of endemic species (Baser, 2002), Turkish

propolis samples being discrete from those typical for Central and Eastern Europe.

In the present study, according to subjective visual classification, 37 propolis samples were assigned to the O-type, 18 of them to the B-type and 5 to the new third type (Supplemental Table S1). Among the investigated Turkish propolis samples, O-type was found to be predominant type and showed presence of almost all investigated standards. Characteristic orange band with hR_F range at 29 was recognized as quercetin (Figure 2a). Furthermore, blue and greenish fluorescent bands with hR_F values at 9, 38, 54, 62, 75, 79, 88 were assigned to apigenin, chrysin, caffeic acid, naringenin, CAPE together with pinobanksin, galangin and pinocembrin, respectively (Figure 2a). On the otherside, B-type was characterized by only light blue or green bands corresponding to previously mentioned compounds, without orange band. These compounds were also reported by Bertrams et al. and Morlock et al. (Bertrams et al., 2013; Morlock et al., 2014). Also, Ristivojevic et al. identified galangin, CAPE, chrysin and pinocembrin as characteristic markers of the *P. nigra* buds which was proven to be the main botanical source of O-type propolis (Ristivojević et al., 2014). O-type propolis was also found to be the predominant type in samples from Serbia. Due to the determined similarity between the Turkish and Serbian propolis samples, we can assume that the botanical origin of O-type propolis from these two European countries were same, i.e. black poplar type (Supplemental Figure S1).

With regard to the new third type, in the samples P14, P27, P30 and P42 from Turkey, characteristic bands corresponding to phenolic compounds were absent. Considering the rich terpenoid content in Brazilian, Greek or Cretan type propolis, i.e. monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids, we assumed that the major components in the third type propolis might be terpenes rather than phenolics (Huang, Zhang, Wang, Li, &

Hu, 2014; Kartal, Kaya, & Kurucu, 2002; Popova, Chinou, Marekov, & Bankova, 2009; Popova, Graikou, Chinou, & Bankova, 2010). Due to the fact that terpenic compounds have affinity to apolar solvents, such as chloroform, they were additionally extracted with chloroform and analyzed under conditions characteristic for terpenic compounds (Reich & Schibli, 2007). Consequently, vast number of bands which could be attributed to terpenes were obtained on the chromatograms (Figures 2c and d). However, in order to reliably confirm the chemical profile of these few samples further investigations on higher number of carefully selected representative samples are required.

3.2. HPTLC profile of Turkish propolis vs. HPTLC profile of bud extracts

In order to define accurately the botanical origin of Turkish propolis, chemical profiling of secondary plant metabolites in propolis samples were compared with the characteristic metabolites in the bud extracts of *Populus* spp. (*P. nigra, P. tremula, P. alba*), *Aesculus hippocastanum, Betula pendula, Quercus petraea, Salix caprea, Ulmus glabra, Tilia* spp. (*T. americana, T. tomentosa, T. europaea*) by HPTLC (Figure 3).

HPTLC analysis has shown a great diversity in the phenolic profiles of these plant resins. *P. nigra* bud extract demonstrated a rich phenolic profile with several intensive orange, blue and light green bands. Although of different geographical origin, chromatograms of *P. nigra* from Serbia and Turkey indicated almost identical profiles. Phenolic profile without orange bands and with smaller amount of light and intensive blue bands with lower hR_F ranges were observed in the *P. tremula* extract. The absence of the yellow and orange bands in *P. tremula* was also confirmed by Morlock et al. and Berthrams et al. (Bertrams et al., 2013; Morlock et al., 2014). Several blue and greenish bands were detected on *A. hippocastanum* chromatogram, also confirmed by Berthrams et al. (Bertrams et al., 2013), while only one

bluish band was observed on *Q. petraea* chromatogram. Moreover, chromatogram of *B. pendula* contained several intensive green and light blue bands as previously reported by Berthrams et al. (Bertrams et al., 2013). Mentioned blue and greenish bands do not match with those found in *P. nigra*. All other samples showed profiles without visible zones under the experimental conditions indicating absence of phenolics.

The HPTLC profiles of plant resins, as potential botanical sources of propolis, were compared with profiles of Turkish propolis extracts. Based on phenolics fingerprint, O-type of Turkish propolis showed similarity with *P. nigra* buds, while B-type has similar HPTLC pattern with *P. tremula* buds. These results were in agreement with Berthrams et al. (Bertrams et al., 2013). On the other hand, the new third type of Turkish propolis did not show similarity with any of the *Populus* species. *P. nigra* extract was also observed to contain characteristic bands of apigenin, quercetin, chrysin, caffeic acid, naringenin, CAPE, pinobanksin, galangin, and pinocembrin, which is identical with O-type of propolis, while *P. tremula* was found to contain only caffeic acid and naringenin, similar to B-type (Figure 3). The HPTLC profiles of other nonpoplar plants did not show further strong coherence with orange and blue types of Turkish propolis.

3.3. HPTLC fingerprint for the assessment of authenticity of Turkish propolis samples

HPTLC phenolic profiles of Turkish propolis samples in combination with multivariate image analysis and pattern recognition technique were used for the assessment of their botanical and geographical origin. In order to recognize characteristic markers of botanical and geographical origin, to confirm the presence of botanically different types of Turkish propolis, and to find similarity/dissimilarity between Turkish and Serbian propolis samples, PCA, as the most commonly used pattern recognition technique, was applied. PCA reduces the

multidimensional data set mainly into the two or three dimensions transforming the original variables (intensities of pixels along the 427 length lines) into the new, uncorrelated variables known as principal components (PCs) (Varmuza & Filzmoser, 2008).

First, PCA was used to seek for some logical patterns in the data that might explain botanical origin of Turkish propolis samples. The obtained four-component model explained 75.10% of total variance, 35.22% of the overall data variances was accounted by the first principal component (PC1), and 20.79% by the second one (PC2). Mutual projections of factor scores and their loadings for the first two PCs have been presented in Figures 4a and 4b. Taking into account PC1 and PC2 score values (Figures 4a and 4b) three groups of samples belonging to three different botanical origins of propolis samples (O-, B- and third types) were obtained. There was some overlapping Hotelling T^2 ellipses among O- and B-type propolis samples. In spite of that, third type samples were distant from other two groups and firmly clustered, exhibiting small internal variability. The most influential variables responsible for differentiation of propolis according to botanical origin as well as potential marker compounds for Turkish propolis were identified using the loading plots. The loading plot (Figures 4c and 4d) indicates correlations between the variables and the PCs. Phenolic compounds negatively correlated with PC1 were compounds with hR_F values at 29 (quercetin), 43, 54 (caffeic acid), and 70, while PC2 was affected by phenolic compounds with hR_F values at 9 (apigenin), 29 (quercetin), 43, 54 (caffeic acid), 62 (naringenin), 67, 75 (CAPE together with pinobanksin) and 79 (galangin). Fluorescence blue-greenish band recognized as important marker for botanical origin of Turkish propolis with hR_F range at 43 could be identified as kaempferol. These phenolics are important for discrimination of propolis types. These results are in agreement with studies on German propolis, in which

these compounds were recognized as markers for the B- and O-type propolis (Bertrams et al., 2013; Morlock et al., 2014). To get better insight into differentiation between O- and B-type of Turkish propolis according to their phenolics profiles third type samples were excluded from multivariate analysis. The obtained separation among two subtypes were confirmed alongside the PC1 direction (Figure 4b). The samples of two varieties are dissipated in a broader range of the score space and some overlapping of samples that occurred could be expected due to the complex composition of propolis samples that contained vast number of plant buds resins.

In order to find specific grouping of Turkish and Serbian propolis samples according to their geographical origin, PCA was applied on data obtained from chromatographic profiles. The PCA resulted in a three-component model that explained 81.85% of total variance. The PC accounted for 60.63%, PC2 for 12.30% and third principal component (PC3) for 9.53% of the overall variance. Mutual projections of factor scores and their loadings for the first three PCs have been presented in Figure 5a. The 3D score plot revealed that samples were not firmly clustered according to their geographical origin, i.e. Turkish and Serbian samples were not clearly distinguished. Although both were from the same botanical origin that correspond to European poplar type, certain differences arising from the geographical and climatic conditions have still existing. Loading plots again confirmed the importance of phenolics such as caffeic acid (hR_F at 54), CAPE together with pinobanksin (hR_F at 75), galangin (hR_F at 79), and compounds with hR_F at 30, 43 and 47 (Figures 5b, c and d).

To conclude, pattern recognition technique confirms the presence of three botanically different types of Turkish propolis as well as several subtypes according to their geographical origins. Above mentioned compounds were recognized as most important phenolic markers

for characterization of Turkish propolis.

3.4. Microscobic analysis of Turkish propolis samples

Pollen grains come from anemophilous or entomophilous owers adhere to the resin during its collection by the bees or from harvested pollen inside the beehives (Barth & Fernandes Pinto da Luz, 2003). Identification of the pollen content in propolis samples may provide further evidence for characterization of the vegetation at the vicinity of beehive and the geographical origin of propolis (Barth & Fernandes Pinto da Luz, 2009). The diversity of the plant species used by the bees to prepare propolis may be an indication for acceptable quality and wider medicinal properties. However, numbers of such palynological studies on Turkish propolis are scarce (Çelemli & Sorkun, 2012; Gençay & Sorkun, 2006).

A great variety of pollen types were detected in the propolis samples (Supplemental Figure S3). The pollen spectra were consisted of both nectar and non-nectar plant pollen grains. The results were listed in Table S2 (Supporting information) in accordance with criteria applied in melissopalynology (Louveaux, Maurizio, & Vorwohl, 1970). Pollen frequency was indicated as: DP for dominant (more than 45%), SP for secondary pollen (between 16-45%), while pollens which are significant to characterize the phytogeographical origin of the sample and constitute only 3-15% of pollen grains was marked as important minor pollen (IMP). However, the last group, minor pollen (MP), comprising less than 3% of the pollen grains, includes a great number of less important plant species, sometimes represented by only a single grain, is not presented in Supplemental Table S2.

Pollen grains belonging to 52 family and 75 taxa were identified in Turkish propolis samples. Among these, particularly those belonging to Asteraceae, Fabaceae, Rosaceae, Brassicaceae, Umbelliferae, Plantaginaceae, Salicaceae, Fagaceae, Poaceae, Lamiaceae, Polygonaceae and

Rhamnaceae families were detected almost in all samples (Supplemental Figure S3). The possible plant sources of pollen grains in propolis samples were also identified: Pinaceae (*Abies* spp., *Pinus* spp.), *Acer* spp., Betulaceae (*Betula* spp., *Alnus* spp., *Carpinus* spp., *Corylus* spp.), Fagaceae (*Castanea sativa* Mill., *Quercus* spp.), *Eucalyptus* spp., *Prunus spinosa*, Salicaceae (*Salix* spp., *Populus* spp.), *Tilia* spp., *Ulmus* spp.

In previously published studies on propolis samples, Warakomska and Maciejewicz (Warakomska & Maciejewicz, 1992) and Moreira et al. (Moreira et al., 2008) reported the presence of pollen grains of *Populus* spp. from Polish samples, while Gençay and Sorkun did not evaluate *Populus* spp. in propolis samples collected from east Anatolia (Turkey) (Gençay & Sorkun, 2006). In addition, Çelemli and Sorkun observed only minor amount of them in propolis samples from Tekirdag (Turkey) (Çelemli & Sorkun, 2012). In the present study, *Populus* spp. pollens have not been determined in any of the samples. Possible explanation for this incoherent fact that honeybees mostly collect the sticky secretions on the surfaces of *Populus* spp. buds just before flowering period, and accordingly pollen grains could not be detected.

On the other hand, the type of several propolis samples (i.e. P4, P7, P20, P24, P37, P42, P45, P46 and P59) were not characterized on the basis of pollen analysis, since neither dominant (DP) nor secondary pollen (SP) grains were observed. Therefore, applications merely based on pollen analysis would not be a satisfactory approach for determination of botanical origin of a propolis sample. Briefly, pollen analysis gives only preliminary information about the most dominating botanical species in the vicinity of the beehive, but not for determination of the propolis type. Therefore, in order to confirm the botanical origin of propolis precisely, chromatographic fingerprint analysis should be performed simultaneously with plant bud

extracts.

3.5. Antioxidant activity of Turkish propolis evaluated by HPTLC-DPPH˙ analysis

DPPH˙ assay combined with HPTLC is a fast screening technique that provides identification of each phenolic component with potential antioxidative activity. Antioxidant activity of compounds separated on the plate could be identified by detection of yellow color bands against purple background obtained after dipping of HPTLC chromatogram in DPPH˙ solution.

The HPTLC chromatograms of propolis samples after treatment with DPPH˙ reagent showed colored bands with hR_F values that correspond to caffeic acid, galangin, CAPE and pinobanksin (Figure 2b). Identical activity of individual compounds in the standard mixture was also confirmed (the concentration of chrysin, caffeic acid, CAPE and pinobanksin 307.5, 307.5, 307.5 and 0.615 μg/band, respectively). The antioxidant activity of propolis samples was probably resulting from the presence of phenolic components possessing *o*-dihydroxy phenyl structure which is the basic structural requirement for significant radical scavenging activity. Namely, caffeic acid, quercetin, kaempferol, galangin, phenethyl caffeate, cinnamyl caffeate were reported as the main phenolics in propolis with high reducing power (Kumazawa, Hamasaka, & Nakayama, 2004).

For the O-type of Turkish propolis, components with hR_F ranges at 29, 47, 54, and 75 in the area of pronounced blue bands displayed higher reducing capacity. Contrarily, the components having blue fluorescence in the B-type of propolis exhibited weak antioxidative power. This finding is in accordance with results reported by Bertrams et al., where O-type German propolis showed higher reducing capacity comparing to B-type (Bertrams et al., 2013). On the other hand, the third new type of propolis which was explored in this study did

not show any reducing power against DPPH˙ reagent.

The applied quantitative method in this study is based on calculation of total extract area using Camag VideoScan TLC evaluation software which converts images into chromatograms. The sum of all the peaks (the total area of one propolis extract) was considered as the total antioxidant activity of all separated compounds on the chromatogram. When total antioxidant capacity of all propolis samples were compared, the sample encoded P11, which was provided from Persembe (Ordu) was found to exert the highest antioxidant activity among all samples. According to the HPTLC fingerprinting, this sample was an O-type propolis possibly originating from *P. nigra*. When the result of pollen analysis of P11 was examined, the highest pollen count was determined to belong to chestnut (*C. sativa*) in the ratio of 88% (Supplemental Table S2).

This is a particularly important outcome demonstrating the influence of environmental plant diversity around the beehive in the chemical composition and thus pharmaceutical properties of propolis. Accordingly, disposition of beehives where *P. nigra* and *C. sativa* tree populations are abundant would possibly increase the pharmaceutical value of propolis.

4. Conclusion

Turkey is characterized by widely varying climatic and topographic conditions, which result in a broad diversity in forest ecosystems, species and within-species variation. Consequently a widerange of diversity are expected among the propolis samples. In the present study, authentication of Turkish propolis samples according to their HPTLC phenolic fingerprinting and palynological profiles were performed. In addition to known two main propolis types (O- and B-types) from different botanical origins, a new third type propolis was discovered first time here. O-type of Turkish propolis originated from *P. nigra*, while B-type showed a similar

pattern as *P. tremula* buds. Application of HPTLC profiling hyphenated with pattern recognition technique for the assessment of botanical and geographical origins of Turkish propolis were also confirmed in comparison to bud resins for botanical origin, *i.e.* Serbian propolis, as a representative of European propolis, for geographical origin. According to the pollen analysis, *C. sativa* proved to be one of the dominant pollen grains found in the propolis samples. It should be emphasized that performing only pollen analysis is not a satisfactory approach to determine the botanical origin of propolis, due to the fact that most of the samples, as well as those analyzed in this study, are lack of dominant pollen and even secondary pollen grains. Therefore, authentication of propolis should primarily be performed by chromatographic analysis and then palynological profiles may be examined as a secondary tool.

Acknowledgements

Etil Guzelmeric is grateful to Pharm. Fatih Cengiz and Pharm. Rizahan Sarigoz for their contributions to the study. She also thanks to the members of Turkish Association of Beekeepers for supplying propolis samples. In addition, this work has been supported by the Ministry of Education, Science and Technological Development of Serbia, Grants No. 172017.

References

Bankova, V. (2005). Chemical diversity of propolis and the problem of standardization. *Journal of Ethnopharmacology*, *100*, 114–117.

Barth, O.M. (1998). Pollen analysis of Brazilian propolis. *Grana*, *37*, 97-101.

Barth, O.M., & Fernandes Pinto da Luz, F. (2003). Palynological analysis of Brazilian geopropolis sediments. *Grana*, *42*, 121–127.

506 Barth, O.M., & Fernandes Pinto da Luz, C. (2009). Palynological analysis of Brazilian red
507 propolis samples. *Journal of Apicultural Research, 48,* 181-188.
508 Başer, K.H.C. (2002). Aromatic biodiversity among the flowering plant taxa of Turkey. *Pure*
509 *and Applied Chemistry, 74,* 527-545.
510 Berthrams, J., Müller, M.B., Kunz, N., Kammerer, D.R., & Stintzing, F.C. (2013). Phenolic
511 compounds as marker compounds for botanical origin determination of German
512 propolis samples based on TLC and TLC-MS. *Journal of Applied Botany and Food*
513 *Quality,86,* 143-153.
514 Bertelli, D., Papotti, G., Bortolotti, L., Marcazzan, G.L., & Plessi, M. (2012). 1H-NMR
515 Simultaneous identification of health-relevant compounds in propolis extracts.
516 *Phytochemical Analysis, 23,* 260-266.
517 Boisard, S., Le Ray, A-M., Landreau, A., Kempf, M., Cassisa, V., Flurin, C., & Richomme, P.
518 (2015). Antifungal and Antibacterial Metabolites from a French Poplar Type Propolis.
519 *Evidence-Based Complementary and Alternative Medicine,* Article ID 319240, 10
520 pages, http://dx.doi.org/10.1155/2015/319240
521 Candolfi, A., Maesschalck, R. De, Jouan-Rimbaud, D., Hailey, P.A., & Massart, D.L. (1999).
522 The influence of data pre-processing in the pattern recognition of excipients near-
523 infrared spectra. *Journal of Pharmaceutical and Biomedical Analysis, 21,* 115–132.
524 Cao, Y.H., Wang, Y., & Yuan, Q. (2004). Analysis of flavonoids and phenolic acid in
525 propolis by capillary electrophoresis. *Chromatographia, 59,* 135-140.
526 Castaldo, S., & Capasso, F. (2002). Propolis, an old remedy used in modern medicine.
527 *Fitoterapia,* 73, 1-6.
528 Cieśla, Ł., Kryszeń, J., Stochmal, A., Oleszek, W., & Waksmundzka-Hajnos, M. (2012).

529 Approach to develop a standardized TLC-DPPH· test for assessing free radical
530 scavenging properties of selected phenolic compounds. *Journal of Pharmaceutical*
531 *and Biomedical Analysis*, *70*, 126-135.
532 Çelemli, Ö.G., & Sorkun, K. (2012). The plant choices of honey bees to collect propolis in
533 Tekirdağ-Turkey. *Hacettepe Journal* of *Biology* and *Chemistry*, *40*, 45-51.
534 Duran, N., Muz, M., Culha, G., Duran, G.,& Ozer, B. (2011). GC-MS analysis and
535 antileishmanial activities of two Turkish propolis types.*Parasitology Research 108*,
536 95-105.
537 Erdtman, G. (1952). *Pollen Morphology and Plant Taxonomy-Angiosperms*. US: Chronica
538 Botanica Company.
539 Falcão, S.I., Tomás, A., Vale, N., Gomes, P., Freire, C., & Vilas-Boas, M. (2013). Phenolic
540 quantification and botanical origin of Portuguese propolis. *Industrial Crops and*
541 *Products*, *49*, 805-812.
542 Gençay, Ö., & Sorkun, K. (2006). Microscopic analysis of propolis samples collected from
543 East Anatolia (Kemaliye-Erzincan). *Journal of Pharmaceutical Sciences,31*, 192-197.
544 Ghisalberti, E. L. (1979). Propolis: A Review. *Bee World*, *60*, 59-84.
545 Greenaway, W., Scaysbrook, T., & Whatley, F.R. (1987). The analysis of bud exudate of
546 *Populus* x *euramericana*, and of propolis, by gas chromatography-mass spectrometry.
547 *Proceedings of the Royal Society of London B*, *232*, 249–272.
548 Huang, S., Zhang, C., Wang, K., Li, G.Q., & Hu, F. (2014). Recent advances in the chemical
549 composition of propolis. *Molecules*, *19*, 19610-19632.
550 Isla, M.I., Paredes-Guzman, J.F., Nieva-Moreno, M.I., Koo, H., & Park, Y.K. (2005). Some
551 chemical composition and biological activity of Northern Argentine propolis. *Journal*

552 *of Agricultural and Food Chemistry,53*, 1166-1172.

553 Kartal, M., Kaya, S., & Kurucu, S. (2002). GC-MS analysis of propolis samples from two
554 different regions of Turkey. *Zeitschrift fur Naturforschung C, 57*, 905–909.

555 Kumazawa, S., Hamasaka, T., & Nakayama, T. (2004). Antioxidant activity of propolis of
556 various geographic origins. *Food Chemistry, 84*, 329-339.

557 Kumova, U., Korkmaz, A., Avcı, B.C., & Ceyran, G. (2002). An important bee product:
558 Propolis. *Uludag Bee Journal*, *2*, 10-24.

559 Louveaux, J., Maurizio, A., & Vorwohl, G. (1970). Methodik der Melissopalynologie.
560 *Apidologie*, *1*, 193-209.

561 Milojković-Opsenica, D., Ristivojević, P., Trifković, J., Vovk, I., Lušić, D., & Tešić, Ž.
562 (2016). TLC fingerprinting and pattern recognition methods in the assessment of
563 authenticity of Poplar-type propolis. *Journal of Chromatographic Science*, *54*, 1077-
564 1083.

565 Moreira, L., Dias, L.G., Pereira, J.A., & Estevinho, L. (2008). Antioxidant properties, total
566 phenols and pollen analysis of propolis samples from Portugal. *Food and Chemical*
567 *Toxicology*, *46*, 3482-3485.

568 Morlock, G.E., Ristivojević, P., & Chernetsova, E.S. (2014). Combined multivariate data
569 analysis of high-performance thin-layer chromatography fingerprints and direct
570 analysis in real time mass spectra for profiling of natural products like propolis.
571 *Journal of Chromatography A*, *1328*, 104-112.

572 Morlock, G., Scholl, I., Kunz, N., & Schroeder, A. (2013). Planar-chromatographic
573 fingerprint of German propolis. *CBS 111*, 13-15.

574 Nie, P., Xia, Z., Sun, D. W., & He, Y. (2013). Application of visible and near infrared

spectroscopy for rapid analysis of chrysin and galangin in Chinese propolis. *Sensors*, *13*, 10539–10549.

Park, Y.K., Alencar, S.M., & Aguiar, C.L. (2002). Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry*, *50*, 2502-2506.

Popova. M,, Silici, S., Kaftanoglu, O., Bankova, V. (2005). Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. *Phytomedicine*, *12*, 221-8.

Popova, M.P., Chinou, I.B., Marekov, I.N., & Bankova, V.S. (2009). Terpenes with antimicrobial activity from Cretan propolis. *Phytochemistry,70*, 1262-1271.

Popova, M.P., Graikou, K., Chinou, I., & Bankova, V.S. (2010). GC-MS profiling of diterpene compounds in Mediterranean propolis from Greece. *Journal of Agricultural and Food Chemistry*, *58*, 3167-3176.

Reich, E., & Schibli, A. (2007). *High Performance Thin Layer Chromatography for the Analysis of Medicinal Plants*. New York: Thieme.

Ristivojević, P., Andrić, F.Lj., Trifković, J.Đ., Vovk, I., Stanisavljević, L.Ž., Tešić, Ž.Lj., & Milojković-Opsenica, D. (2014). Pattern recognition methods and multivariate image analysis in HPTLC fingerprinting of propolis extracts. *Journal of Chemometrics,28*, 301-310.

Ristivojević, P., Trifkovic, J., Andrić, F., & Milojkovic-Opsenica, D. (2015). Poplar type propolis: chemical composition, botanical origin and biological activity. *Natural Product Communications*, *10*, 1869–1876.

Salatino, A., Teixeria, Ê.W., Negri, G., & Message, D. (2005). Origin and chemical variation of Brazilian propolis. *Evidence-Based Complementary and Alternative Medicine,2*,

598 33-38.

599 Sârbu, C., & Moţ, A.C. (2011). Ecosystem discrimination and fingerprinting of Romanian
600 propolis by hierarchical fuzzy clustering and image analysis of TLC patterns. *Talanta*,
601 85, 1112-1117.

602 Sawyer, R. (1988). *Honey Identification*. UK: Cardiff Academic Press.

603 Shi, H., Yang, H., Zhang, X., & Yu, L. (2012). Identification and quantification of
604 phytochemical composition and anti-inflammatory and radical scavenging properties
605 of methanolic extracts of Chinese propolis. *Journal of Agricultural and Food*
606 *Chemistry*, *60*, 12403-12410.

607 Sorkun, K., Süer, B., Salih, B. (2001). Determination of chemical composition
608 of Turkish propolis. *Zeitschrift für Naturforschung C*, *56*, 666-8.

609 Varmuza, K., & Filzmoser, P. (2008). *Introduction to Multivariate Statistical Analysis in*
610 *Chemometrics*. US: CRC Press Taylor & Francis Group.

611 Warakomska, Z., & Maciejewicz, W. (1992). Microscopic analysis of propolis from Polish
612 regions. *Apidologie*, *23*, 277-283.

613 Yesilada, E. (2015). *Apiterapi*. Istanbul: Hayykitap.

614

Table 1. List of standard compounds with band colors and hR_F values

Band	**Compound**	**Band color**	$\boldsymbol{hR_F}$
1	Apigenin	green	9
2	Quercetin	orange	29
3	Chrysin	green	38
4	Caffeic acid	blue	54
5	Naringenin	green	62
6+7	CAPE+Pinobanksin	blue	75
8	Galangin	blue	79
9	Pinocembrin	blue	88

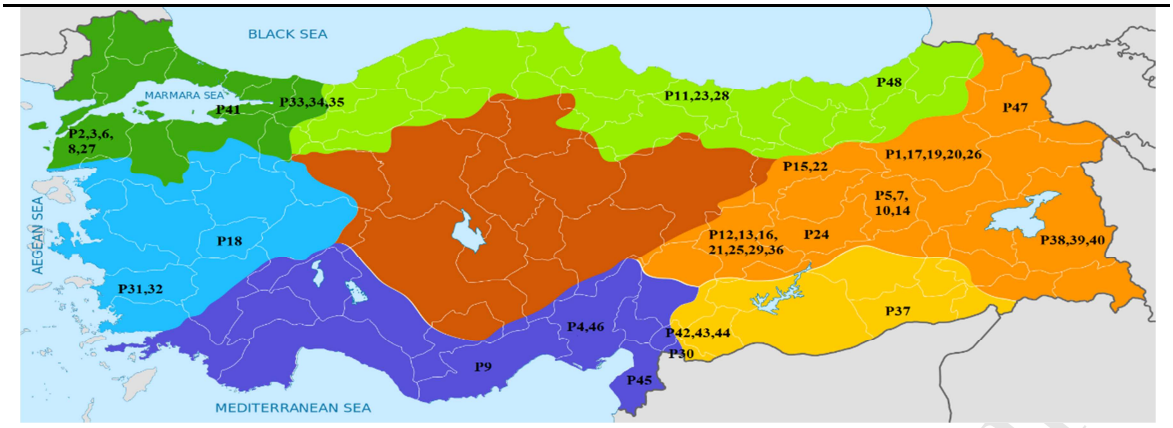

Figure 1. Propolis samples collected from Turkey

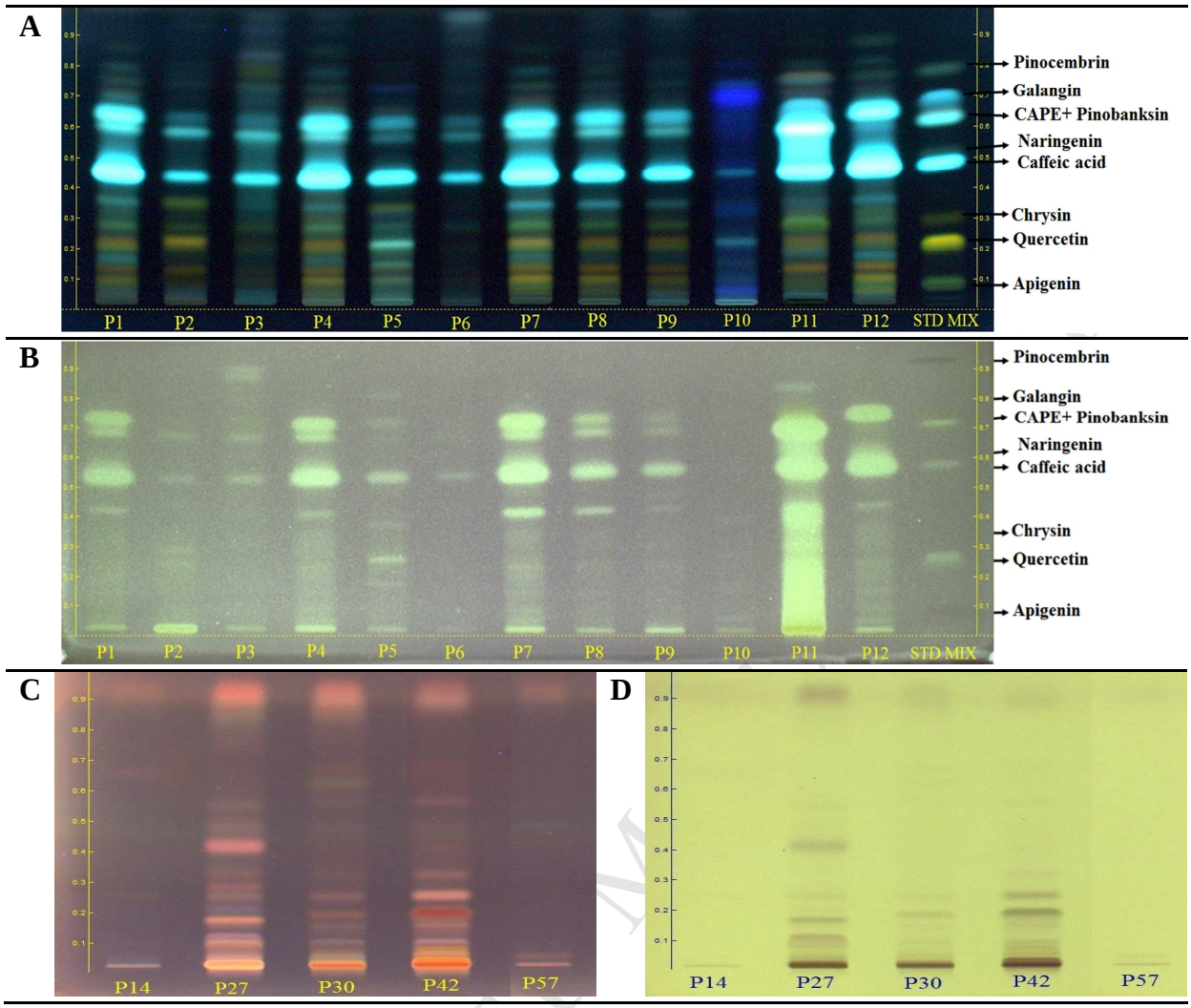

Figure 2. HPTLC chromatograms of (a) hydroalcoholic propolis extracts at 366 nm, developing solvent system: *n*-hexane-ethyl acetate-acetic acid (5:3:1, v/v/v), derivatization: NP/PEG 400; (b) hydroalcoholic propolis extracts at white light, developing solvent system: *n*-hexane-ethyl acetate-acetic acid acid (5:3:1, v/v/v), derivatization: DPPH˙ solution; propolis chloroform extracts at (c) 366 nm and (d) white light, developing solvent system: toluene-ethyl acetate (95:5, v/v/v), derivatization: Anisaldehyde reagent.

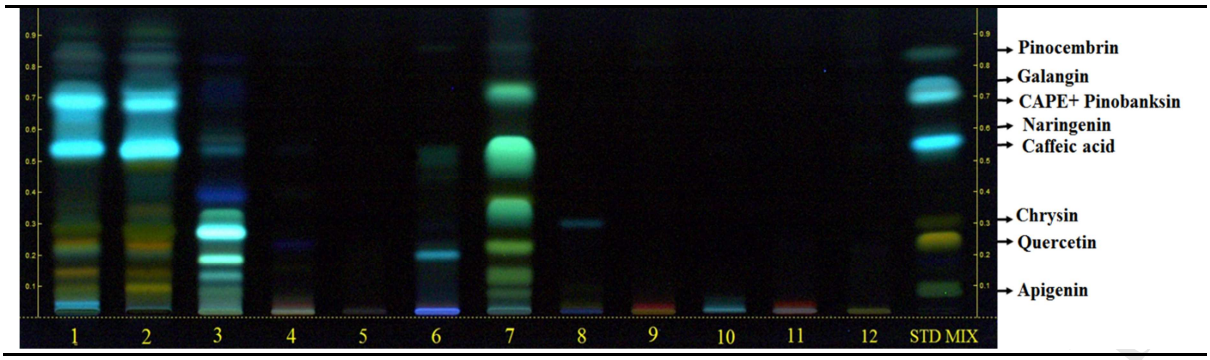

Figure 3. HPTLC chromatogram of plant bud extracts at 366 nm. Track 1 and 2: *P. nigra*; Track 3: *P. tremula*; Track 4: *P. alba*; Track 5: *T. americana*; Track 6: *A. hippocastanum*; Track 7: *B. pendula*; Track 8: *Q. petraea*; Track 9: *S. caprea*; Track 10: *U. glabra*; Track 11: *T. tomentosa*; Track 12: *T. europaea*; developing solvent system: *n*-hexane-ethyl acetate-acetic acid (5:3:1, v/v/v), derivatization: NP/PEG 400.

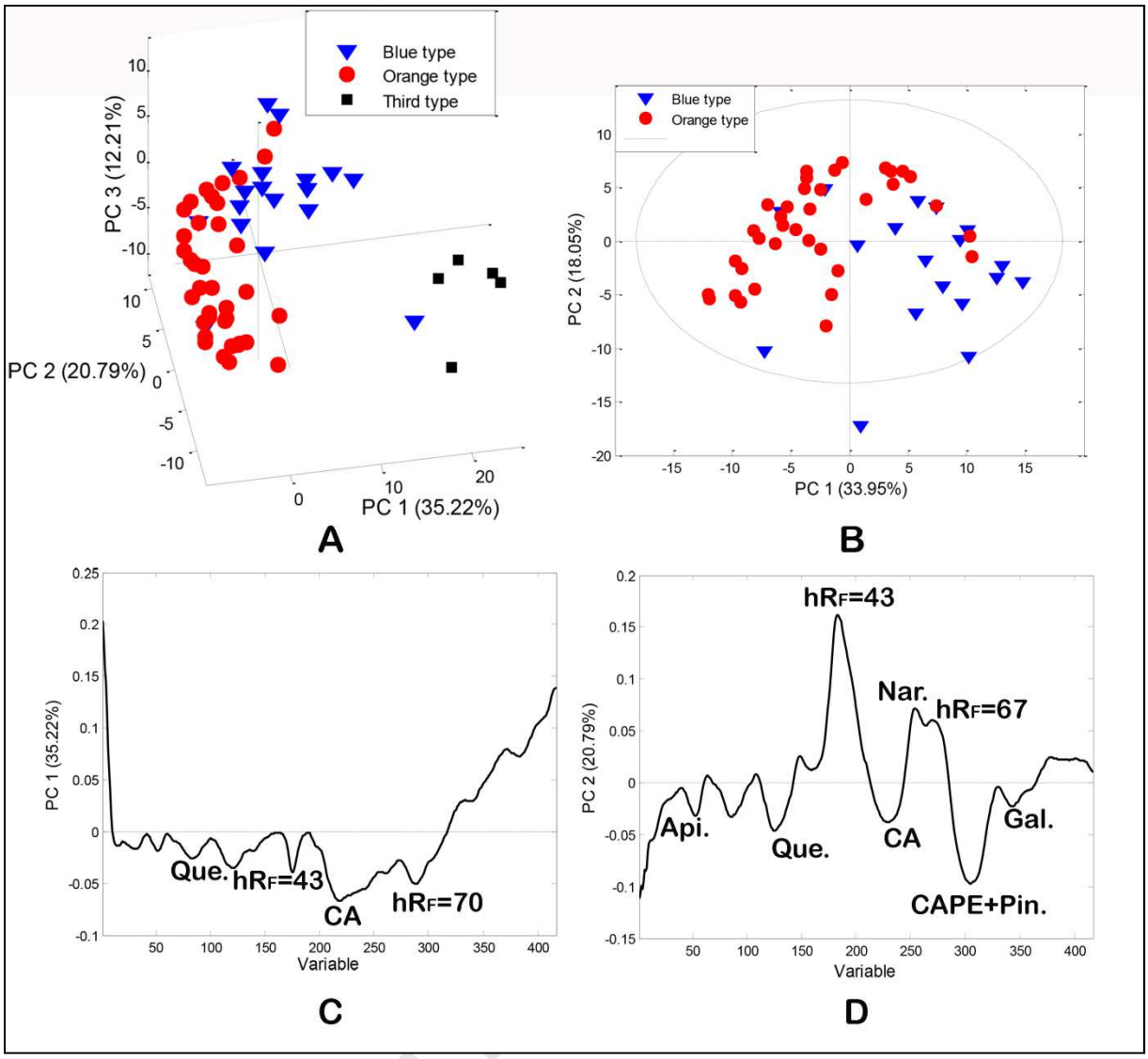

Figure 4. Principal component analysis to determine botanical origin of Turkish propolis: a,b) mutual projections of factor scores, c) loadings for the PC1, d) loadings for the PC2.

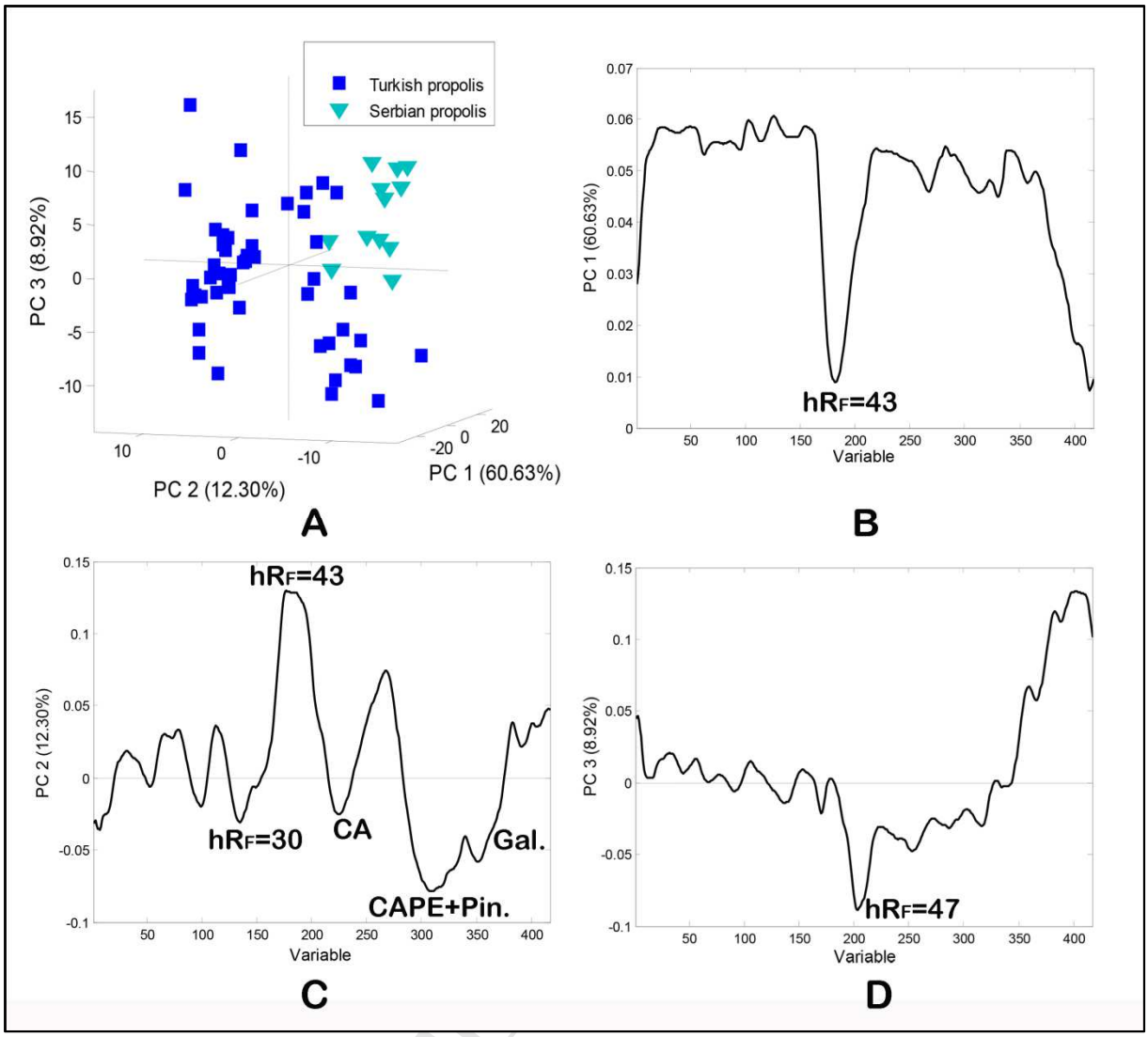

Figure 5. Principal component analysis to determine geographical origin of Turkish and Serbian propolis: a) mutual projections of first three PCs, b) loadings for the PC1, c) loadings for the PC2, d) loadings for the PC3

Highlights

- Turkish propolis was classified as orange, blue and nonphenolic types by HPTLC.
- O-type of propolis originates from *Populus nigra,* while B-type from *P. tremula* buds.
- O-type of propolis exerted higher antioxidant activity than the other propolis types.
- *Castanea sativa* was one of the dominant pollen grains found in the propolis samples.
- Quercetin, caffeic acid, CAPE, pinobanksin and galangin had antioxidant activity.