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PHENOLIC FINGERPRINT OF MACEDONIAN PROPOLIS[1]

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Propolis is a chemically complex resinous material collected by honeybees (*Apis mellifera*) from tree buds and resins, comprising plant exudates, secreted substances from bee metabolism, pollen, and waxes. Its chemical composition depends strongly on the plant sources available around the beehive, which have a direct impact on the quality and bioactivity of the propolis.

In this study, the composition of phenolic compounds in 13 Macedonian propolis extracts was investigated by HPLC-DAD-ESI-MS/MS. Overall, the UV spectra, the MS and MS/MS data allowed the identification of 36 compounds.

The major constituents of propolis were phenolic acids (caffeic and coumaric) and their esters (methyl, (iso)prenyl, benzyl, phenylethyl, cinnamyl), flavonols (quercetin, kaemferol), flavones (chrysin, apigenin, acacetin), flavanonols (pinobanksin), flavanones (pinocembrin, naringenin, hesperetin, pinostrobin) and their methylated/esterified derivatives.

The results reveal that Macedonian propolis contains a diversity of phenolic compounds confirming that it is a poplar type of propolis with higher phenolic content (ranging from 43.75 – 637.94 mg/g) than reported in previous studies in the region and beyond in Europe (< 80 mg/g). This suggests the potential significance of Macedonian propolis as a valuable source of bioactive compounds with health benefits as well as for unlocking its economic potential for industry and beekeepers.

Keywords: propolis; poplar type; HPLC-DAD-ESI-MS/MS; phenolic acids; flavonoids; economic potential

ФЕНОЛЕН ПРОФИЛ НА МАКЕДОНСКИ ПРОПОЛИС

Прополисот е хемиски комплексен смолест материјал кој пчелите (*Apis mellifera*) го собираат од пупките и смолите на дрвјата. Тој е смеса од растителни смоли, супстанции кои се метаболитички продукти на пчелите, полен и восок. Неговиот хемиски состав силно зависи од растителните извори кои се достапни околу пчелните кошници и тие имаат директно влијание врз квалитетот и биоактивноста на прополисот.

Во ова истражување е проучуван составот на фенолните соединенија во 13 македонски екстракти на прополис, со примена на HPLC-DAD-ESI-MS/MS. Со помош на UV-спектрите и податоците добиени од анализата MS и MS/MS во примероците од прополис се идентификувани вкупно 36 фенолни соединенија.

Фенолните киселини (кафена и кумарна) и нивните естери (метил, (изо)пренил, бензил, фенилетил, цинамил), флавонолите (кверцетин, кемферол), флавоните (хризин, апигенин, акацетин), флаванонолите (пинобанскин), флаваноните (пиноцембрин, нарингенин, хесперетин, пиностробин) и нивните метилирани/естерифицирани деривати се доминантни соединенија застапени во проучуваните примероци прополис.

Врз основа на добиените резултати може да се заклучи дека македонскиот прополис се карактеризира со голема разновидност на фенолни соединенија, карактеристични за тополов тип

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на прополис, со повисока содржина на фенолни соединенија (во опсег од 43,75 – 637,94 mg/g) споредено со претходните истражувања на примероци од прополис во регионот и пошироко во Европа (< 80 mg/g). Ова укажува на потенцијално значење на македонскиот прополис како вреден извор на биоактивни соединенија со здравствени придобивки, како и на потенцирање на неговиот економски потенцијал за индустријата и пчеларите.

Клучни зборови: прополис; тополов тип; HPLC-DAD-ESI-MS/MS; фенолни киселини; Флавоноиди; економски потенцијал

1. INTRODUCTION

Propolis is a natural resinous substance produced by bees. Due to its waxy structure and mechanical properties, bees use it to build and repair hives. Propolis serves as protection against potential predators, wind, and water. However, one of the most significant features of propolis is its ability to destroy pathogenic microorganisms, actively reducing the risk of disease in bees and transmission of parasites throughout bee colonies. Propolis is a hard, lipophilic material that becomes soft and very sticky when heated. It has a specific and pleasant aroma, and its color can range from yellow-green to red and even dark brown, depending on the origin and age.[1]

People have been using propolis for a long time due to its positive effects on health, particularly on the immune system. Propolis is known to possess antitumor, antiviral, antibacterial, fungicidal, and various other properties that contribute to health benefits.[2] As a result, propolis finds active use in traditional and modern medicine,[3] as well as in veterinary medicine, pharmacology, and cosmetics,[4] and also as a functional ingredient in foods.[5]

According to previously published data,[6,7] over 800 different compounds have been identified in propolis. The presence of flavonoids such as pinocembrin, galangin, and chrysin, along with phenolic acids such as caffeic, ferulic, and cinnamic acids has also been confirmed. The chemical composition of propolis largely depends on the source plant, resulting in significant variations across different geographical regions. Understanding the chemical composition, the presence of bioactive compounds, and the origin of propolis is a fundamental prerequisite for its chemical standardization.[7]

According to literature,[8] one comes across descriptions of various types of propolis identified by their chemical profiles, including 'Poplar type', 'Birch type', 'Tropical type', 'Mediterranean type' and 'Pacific type'.

The Balkans and the Macedonian region have a long history of producing and using propolis, with records dating back to the first millennium BC. In addition, propolis is authorized as a food supplement and is available on the market in multiple formulations but are prepared with different types of propolis in which the main active compounds are not identified and there is no precise specification on the label nor criteria regarding the doses indicated. The knowledge of the chemical composition, the type and content of bioactive components, and the plant origin of propolis is a basic prerequisite for its chemical standardization. Nevertheless, there are currently very limited published data[9–11] on the specific chemical composition of Macedonian propolis.

The aim of this study is to comprehensively assess propolis samples collected from different regions of the country. The chemical profiling of propolis extracts was conducted by analyzing the content of individual phenolic compounds using HPLC-DAD-ESI-MS/MS. The obtained results are valuable for providing an insight into the nature and number of the detected compounds and their quantity, and consequently for preparation of their commercial formulations. The obtained results are also important for the food industry and for the local beekeepers to explore the economic value of propolis. This understanding could potentially lead to increased production of high quality propolis as a raw product and its application in both simple and complex multicomponent formulations with antibacterial and immunomodulatory activity.

2. MATERIAL AND METHODS

2.1. *Reagents and standards*

HPLC grade formic acid, methanol, acetonitrile and water, were purchased from Merck KGaA (Darmstadt, Germany); 5-caffeoylquinic acid, quercetin and naringenin were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. *Samples*

Samples were supplied by beekeepers in July – August 2020, from the regions of Kumanovo (**MK1**), Kriva Palanka (**MK2**), Makedonska

Kamenica (**MK3**), Delčevo (**MK4**), Tetovo (**MK5**), Kičevo (**MK6**), Resen (**MK7**), Bitola (**MK8**), Skopje (**MK9**), Veles (**MK10**), Negotino (**MK11**), Gevgelija (**MK12**) and Dojran (**MK13**). Full sample details, including collection locations, are included in Table 1. A map of North Macedonia, indicating the locations where the different samples were collected, is shown in Figure S1 in Supplementary material.

T a b l e 1

Propolis samples collection data (codes, location, year)

Sample	Latitude	Longitude	Altitude	Location	Year of collection
MK-1	41°55′38″N	21°46′0.05″E	600 m	s. Divlje, Kumanovo	2020
MK-2	42°12′25.27″N	22°19′40.73″E	600 m	Kriva Palanka	2020
MK-3	42°1′17.18″N	22°35′13.49″E	500 m	Makedonska Kamenica	2020
MK-4	41°58′15.13″N	22°46′26.22″E	500 m	Delčevo	2020
MK-5	42°0′22.75″N	20°56′39.01″E	700 m	Tetovo	2020
MK-6	41°30′40.46″N	20°56′42.36″E	700 m	Kičevo	2020
MK-7	41°5′1.36″N	21°0′50.33″E	800 m	Resen	2020
MK-8	41°1′8.54″N	21°19′29.75″E	600 m	Bitola	2020
MK-9	41°58′50.77″N	21°22′16.97″E	500 m	Skopje	2020
MK-10	41°42′59.22″N	21°46′20.24″E	100 m	Veles	2020
MK-11	41°30′21.02″N	22°15′52.13″E	600 m	s. Kalanjevo, Negotino	2020
MK-12	41°12′15.34″N	22°29′57.91″E	100 m	s. Prdejci, Gevgelija	2020
MK-13	41°14′43.44″N	22°36′47.38″E	300 m	s. Furka, Dojran	2020

2.3. *Extraction and sample preparation*

Frozen propolis was grated and 2 g was dissolved in 40 ml 70 % ethanol in a 100 ml flask and left for 24 h at room temperature and the supernatant was collected (three replicates from each sample). The residue was then re-extracted and the extracts were combined and evaporated to dryness. The dry extract was dissolved in 50 ml methanol and analyzed by HPLC-DAD-ESI-MS/MS. The extract was filtered through a 0.45 μm pore nylon membrane filter before analysis.

2.4. *HPLC-DAD-ESI-MS/MS*

For identification and quantification of phenolic compounds, an HPLC system (Agilent 1100) coupled with a UV-Vis diode-array detector and an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system was used. Chromatographic separations were conducted using a Supelco C18 column (250 × 4.6 mm, 5 μm), with a mobile phase consisting of 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B). The elution was isocratic with 45 % B in the initial 5 minutes, followed by a linear gradient to reach 55 % B at 40 min, continuing with 55 % B isocratic at 50 min, then reaching 70 % B at 70 min, 100 % at 90 min, and subsequently holding 100 % B for the final 10 minutes. The flow rate was 0.3 ml/min and the injection volume was 10 μl.

Spectral data were accumulated in the range 190–600 nm and chromatograms were recorded at 290 and 350 nm for flavonoid derivatives and at 330 nm for phenolic acids.

For MS analysis, an ion-trap mass detector (with an electrospray ionization (ESI) system) was used in negative ionization mode, covering the full scan mass range from *m/z* 100–1200. For the MS detector, nitrogen was utilized as the nebulizing gas at a pressure level of 50 psi with a flow rate of 12 l/min. The capillary temperature and voltage were set at 325 °C and 4 kV, respectively.

For qualitative analysis, both detectors were utilized, and the UV-Vis and MS spectra data were compared with the spectra of available standards or literature data. For quantitative analysis, only the signals obtained from the UV detector were used. All phenolic acids were quantified as caffeic acid equivalents at 330 nm, flavonols and flavones as quercetin equivalents at 350 nm, while flavanonols and flavanones were quantified as naringenin equivalents at 290 nm.

2.5. *Statistical analysis*

Statistical analysis of the data was performed using Excel 2019 for calculations of cali-

bration curves, mean, and standard deviation. Principal component analysis was performed using the software TANAGRA 1.4.28 (Lyon, France).

3. RESULTS AND DISCUSSION

3.1. *Characterization of phenolic compounds*

Identification of phenolic compounds was accomplished by comparing their chromatographic behavior as well as UV-Vis and MS spectra (in negative ionization mode) with available standards and literature data. In all analyzed samples, a total of 36 compounds were detected and quantified, then classified into five groups such as: phenolic acids and derivatives, flavonols, flavones, flavanonols and flavanones. The retention and spectral data for the identified polyphenolic compounds in all 13 collected samples are presented in Table 2. The structures are presented in Figure 1. The typical HPLC-DAD chromatogram obtained for the propolis extract collected from Kriva Palanka (MK-2) is given in Figure 2.

In all analyzed samples, twelve phenolic acid derivatives were identified, primarily classified as caffeic acid derivatives with typical UV spectra showing absorption maxima at 220 and 324 nm and a diagnostic shoulder at 296 nm. Additionally, one *p*-coumaric derivative was observed with an absorption maximum at 312 nm.

Caffeic acid (**Pa1**) and *p*-coumaric acid (**Pa3**) gave a typical product ion at *m/z* 135 and 119, respectively, corresponding to $[M–COO]^-$. Caffeic acid derivatives, such as caffeic acid isoprenyl/prenyl/phenylethyl esters (**Pa2**, **Pa4**, **Pa9** and **Pa10**) gave product ions at *m/z* 179 and 135 corresponding to [M–isoprenyl/prenyl/ phenylethyl]$^-$ and [M–methyl/isoprenyl/prenyl/ phenylethyl–COO]$^-$, respectively. In contrast, the MS/MS of caffeic acid benzyl ester (**Pa8**) and caffeic acid cinnamyl ester (**Pa12**) yielded typical product ions with *m/z* 178 and 134 corresponding to [M–prenyl/cinnamyl]$^{-\bullet}$ and [M–prenyl–COO/cinnamyl]$^{-\bullet}$, respectively. These results arose from homolytic cleavage, leading to the formation of a radical product.

For *p*-coumaric acid, methyl/isoprenyl esters (**Pa6**, **Pa7**) fragment ions at *m/z* 163 and 119 can be observed due to the loss of the methyl/isoprenyl moiety and an additional loss of a COO^- group. Different types of caffeic or *p*-coumaric acid derivatives have previously been identified in propolis samples from Spain,[12] Portugal,[13,14] Greece,[15] Serbia[16] and Bulgaria[17]. The presence of quinic, ferulic, benzoic and ellagic acids has also been reported as typical for propolis samples from the region, but they were not observed in the analyzed samples.

In total, 24 flavonoids were found as aglycones, classified as flavonols, flavones, flavanonols, and flavanones.

Nine flavonols were primarily identified, including quercetin and kaempferol and their methylated derivatives isorhamnetin, kaempferide and isokaempferide. In the negative mode, the loss of groups such as H_2O (−18), CO (−28), C_2H_2O (−42) and CO_2 (−44) are common for all flavonols. Quercetin and kaempferol, as aglycones, are frequently found regardless of the type of propolis.[12,18–20] However, their methyl ethers have been previously reported in propolis samples from Portugal,[13,14] Serbia,[16] and Croatia.[21]

All six flavanonols were identified as pinobanksin derivatives. Compounds **Fnnl1** and **Fnnl2** gave deprotonated molecular ions at *m/z* 285 and 271, leading to fragment ions at *m/z* 267 and 253 $[M–H_2O]^-$ and 239 and 225 $[M–H_2O–CO]^-$, respectively.

According to literature data,[8,16] esterification of pinobanksin predominantly takes place at C-3, and compounds **Fnnl3-6** were identified as pinobanksin-3-*O*-acetate, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate, and pinobanksin-3-*O*-pentanoate, respectively. In MS/MS analysis, each of them produced abundant ions at *m/z* 271, representing [M–acyl group]$^-$ ions, which further yielded ions at *m/z* 253 corresponding to $[M–\text{acyl group}–H_2O]^-$.

Each of these pinobanksin derivatives has been previously identified in propolis samples from Portugal,[13] Serbia,[16] and Spain[12].

Five flavones, including chrysin (and its methyl and methoxy derivatives), apigenin, acacetin, and four flavanones, namely naringenin, hesperetin, and pinostrobin (and one unknown), were also characterized by typical UV and MS spectra that are characteristic of their aglycons. All of them are previously described in propolis.[16,22]

The phenolic composition of the examined samples affirms the identification of all studied samples as belonging to the poplar propolis type, which is the most prevalent and widely distributed type in Europe, North America, non-tropical regions of Asia, New Zealand, and even Africa. The *Populus* species serves as the primary plant source for propolis globally, particularly in temperate regions. Key constituents of poplar propolis include flavonoids lacking B-ring substituents, such as chrysin, pinocembrin, and pinobanksin, along with caffeic acid esters, notably phenethyl ester (CAPE).[23,24]

It should be noted that galangin, considered as a typical flavone of the poplar type of propolis, was

not found in the analyzed samples, which may suggest a peculiar characteristic of Macedonian propolis.

Variations observed in the samples are consistent with the expected fluctuations in resin composition from poplar buds. These differences can be attributed to specific ecological conditions where the trees grow, the timing of propolis collection by bees, and the genetic traits of individual trees.

T a b l e 2

HPLC-DAD-ESI-MS/MS data for polyphenolic compounds identified in propolis samples

	Compounds:	t_R	UV max	$[M–H]^-$	MS^2
	Phenolic acids and derivatives				
Pa1	Caffeic acid*	15.9	198, 220, 244, 296, 324	179	135
Pa2	Caffeic acid isoprenyl ester	17.5	202, 232, 278, 312	247	179
Pa3	*p*-Coumaric acid*	19.9	198, 228, 310	163	119
Pa4	Caffeic acid isoprenyl ester	20.1	200, 220, 242, 296sh, 324	247	179, 135
Pa5	Caffeic acid derivative	26.3	200, 220, 238, 294, 322	285	267, 151
Pa6	*p*-Coumaric acid methyl ester	35.2	200, 308	177	163, 119
Pa7	*p*-Coumaric acid isoprenyl ester	44.3	198, 232, 312	231	163, 119
Pa8	Caffeic acid benzyl ester	52.6	198, 222, 242, 294sh, 324	269	178, 134
Pa9	Caffeic acid prenyl ester	54.5	200, 220, 240, 296sh, 326	247	179, 135
Pa10	Caffeic acid phenylethyl ester	55.4	200, 216, 292, 326	283	179, 135
Pa11	Unknown 1	58.8	198, 216, 226sh, 288, 338	285	139, 145
Pa12	Caffeic acid cinnamyl ester	63.1	200, 216, 248, 294sh, 328	295	178, 134
	Flavonols				
Fnl1	Quercetin*	28.1	202, 256, 372	301	179, 151
Fnl2	Quercetin 4′-methyl-ether*	30.5	202, 256, 268sh, 356	315	299, 253, 245
Fnl3	Kaempferol*	35.3	198, 220, 248sh, 266, 322sh, 366	285	151, 257
Fnl4	Isorhamnetin*	35.5	200, 220, 256, 272, 284, 370	315	299, 253
Fnl5	Kaempferide (Kaempferol-4′-methyl-ether)*	39.1	200, 218, 266, 350	299	283
Fnl6	Quercetin-dimethyl-ether	41.4	202, 254, 268sh, 354	329	313, 299
Fnl7	Isokaempferide (Kaempferol-3-methyl-ether)	42.5	220, 242, 300sh, 328	299	283
Fnl8	Quercetin 3-methyl-ether	46.1	202, 256, 372	315	259, 253, 165
Fnl9	Quercetin-3,4′-dimethyl ether	47.1	202, 256, 268sh, 356	329	313
	Flavones				
Fn1	Techtochrysin	32.3	200, 212, 264, 310	267	251, 223
Fn2	Apigenin*	33.7	200, 220, 266, 338	269	225, 151
Fn3	Acacetin*	43.7	200, 218, 236, 260, 304, 352	283	267, 239
Fn4	Chrysin*	55.6	200, 226sh, 258, 274, 314	253	209
Fn5	Genkwanin	61.2	200, 218, 266, 310, 346	283	267
	Flavanonols				
Fnnl1	Pinobanksin 3-methyl-ether	29.2	196, 218, 286	285	267, 239
Fnnl2	Pinobanksin*	37.4	200, 228, 292, 338sh	271	253, 225
Fnnl3	Pinobanskin-3-*O*-acetate	59.0	218, 230sh, 292, 335sh	313	253
Fnnl4	Pinobanksin-3-*O*-propionate	69.7	200, 222, 230, 294, 328sh	327	253
Fnnl5	Pinobanksin-3-*O*-butyrate	74.4	202, 216, 248, 294, 324	341	253
Fnnl6	Pinobanksin-3-*O*-pentanoate	80.1	198, 214, 292, 340sh	355	253
	Flavanones				
Fnn1	Naringenin*	40.1	200, 218, 282sh, 350	271	125
Fnn2	Hesperetin*	42.3	200, 226, 288	301	165, 135
Fnn3	Pinostrobin*	70.7	218, 242, 290, 324	269	253, 251
Fnn4	Unknown 2	72.6	218, 286, 344	271	253

*The structure was confirmed by the standard substance.

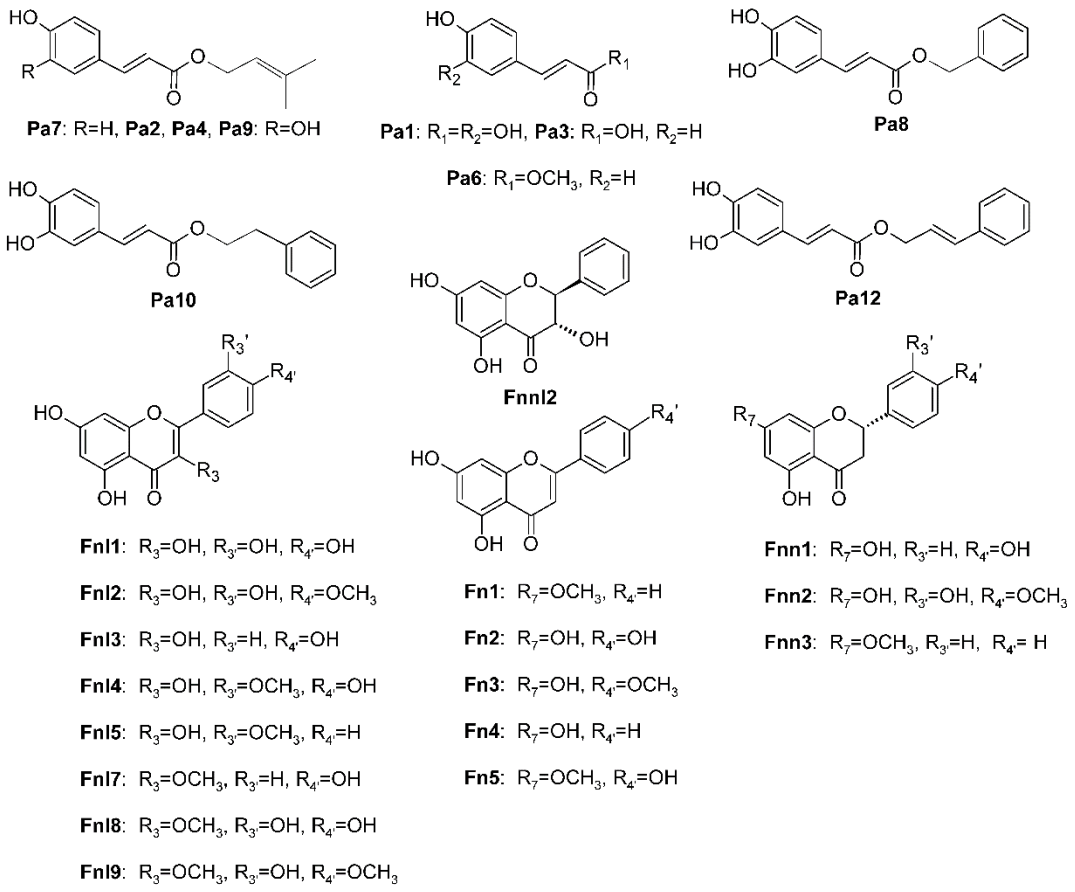


Fig. 1. Chemical structures of phenolic acids and flavonoids detected in analyzed samples. For peak numbers see Table 2.

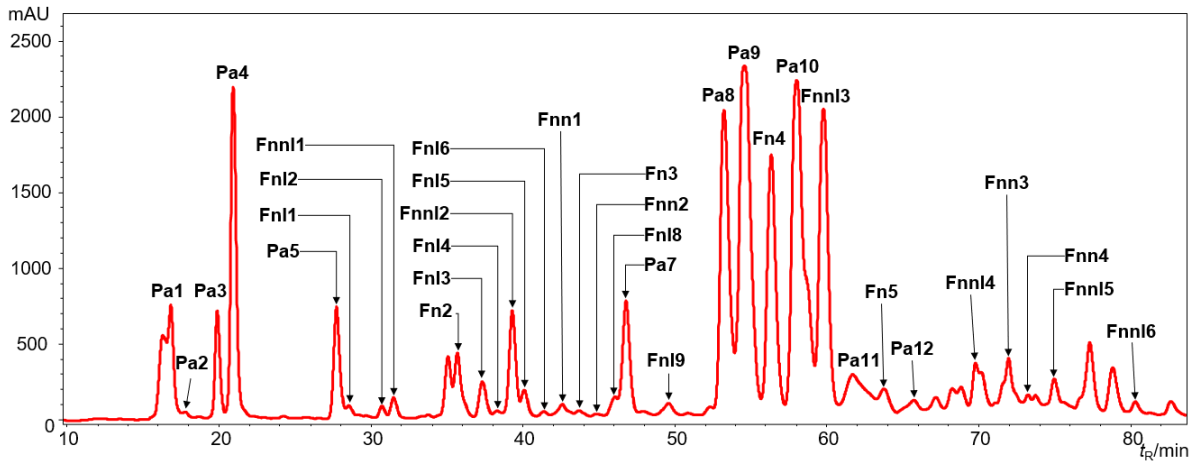


Fig. 2. HPLC-DAD chromatograms for propolis sample collected from Kriva Palanka (MK-2) at 290 nm. For peak numbers see Table 2.

3.2. *Quantitative analysis of phenolic compounds*

All quantitative data for the individual and total polyphenol contents in the studied samples are given in Table 3. The total content of phenolic compounds ranged from 43.75 ± 0.09 mg/g (Gevgelija, MK-12) to 637.94 ± 0.38 mg/g (Kriva Palanka, MK-2) (Table 3).

Phenolic acid derivatives constitute the most abundant group of phenolic compounds, contributing between 30 % and 60 % to the total phenolic content (TPC), followed by flavones (18 – 31 % of TPC). The contribution of pinobanksin derivatives (flavanonols) varies significantly from 4 % to 20 % of TPC. The content of flavonols varies from 2 % to 13 % of TPC. The less abundant group, fla-

vanones, is restricted to 1 – 2 % of TPC, with the exception of MK-11 (Negotino) and MK-12 (Gevgelija), in which their contribution is 8% and 10 % to TPC, respectively. The distribution of each phenolic group to total phenolic content is presented in Figure 3.

The examined propolis extracts exhibited a wide diversity in the concentration of phenolic acids ranging from 27.48 ± 0.18 mg/g (MK-12) to 319.31 ± 1.25 mg/g (MK-2). Caffeic acid prenyl ester (**Pa9**) and caffeic acid phenylethyl ester (**Pa10**) were the most abundant phenolic acid derivatives contributing around 30 % to total phenolic acids content (TPaC). Caffeic acid isoprenyl ester (**Pa4**) accounted for an additional 5 % of TPaC.

Among the flavonoids, flavanonols, that is pinobanksin derivatives were found to be the most abundant group, with concentrations ranging from 2.78 ± 0.01 mg/g in sample MK-12 to 197.51 ± 1.26 mg/g in sample MK-3. Following pinobanksin derivatives, flavones were also present in significant amounts, ranging from 15.49 ± 0.20 mg/g in sample MK-12 to 338.20 ± 7.04 mg/g in sample MK-3. Flavonols were the next most abundant group, with concentrations ranging from 1.68 ± 0.01 mg/g in sample MK-11 to 58.70 ± 0.46 mg/g in sample MK-3.

Comparing the content of different phenolic groups with literature data proves to be challenging due to the different methods used for quantitative analysis. In many published studies, only a limited number of compounds are quantified, and the total phenolic content is expressed as a sum of these individual compounds. However, this approach does not provide a comprehensive picture, as it overlooks the presence of other phenolic compounds in the samples. As a result, quantitative results obtained through such methods may not accurately reflect the true composition and content of phenolic compounds present in the samples.

In that sense, the total content of phenolic compounds in some of the analyzed samples in this study is notably higher compared to those found in literature.

Górecka et al.[25] compared the content of thirteen compounds in the propolis samples from Poland, Turkey, Uruguay and Romania. The results obtained from HPLC analysis range from 4.914 mg/g to 9.218 mg/g. In contrast, the results from spectrophotometric analysis of total phenolic content are in the range from 85.328 mg/g to 155.27 mg/g.

Medana et al.[11] also compared the content of thirteen compounds in propolis samples from various regions. The study reported the following concentrations: Italy (6.243 mg/g), China (9.617 mg/g), Argentina (9.81 mg/g), Ukraine (11.74 mg/g), and Macedonia (15.17 mg/g). This comparison highlights variations in the phenolic compound content among different geographical locations, with Macedonian propolis exhibiting the highest concentration among the regions studied. The content of phenolic compounds is lower compared to the value obtained in our analysis, but this can be justified by the fact that 34 compounds were analyzed and quantified in our paper compared to 13 in the cited paper.

Gardana and Simonetti[10] analyzed the content of caffeic acid derivatives, potential allergens, in propolis samples from various regions. The total content was as follows: Italy (38.2 mg/g), China (53 mg/g), Macedonia (49.9 mg/g), Poland (42.3 mg/g), Uruguay (23.6 mg/g), France (37.4 mg/g), and Nepal (2.68 mg/g) . These findings confirm that the content of caffeic acid derivatives is among the highest across different regions, with Macedonia and China exhibiting particularly elevated concentrations.

The total content of phenolic compounds in the analyzed samples from Greece (sum of 42 compounds)[15] and Poland (sum of 20 compounds)[20] was reported to be up to 40 mg/g and 80 mg/g, respectively.

The results from the phenolic content indicate that the propolis samples present in the region of North Macedonia are characterized by different groups of phenolic acids and flavonoids, and their content is higher compared to similar studies from the region and beyond in Europe.

In order to evaluate the significance of the nature and content of polyphenolic compounds and to explore any correlations and/or distinctions between the studied samples, principal component analysis (PCA) was applied. The PCA analysis applied to the data set revealed five principal components. The first factor (PC1), which explained 44.28 % of the variance, was mainly linked to caffeic acid (**Pa1**) and its phenylethyl (**Pa10**) and cinnamyl (**Pa12**) esters and *p*-coumaric acid (**Pa3**), followed by quercetin (**Fnl1**, **Fnl2** and **Fnl6**) and pinobanksin (**Fnnl1-6**) derivatives. The second principal component (PC2), which explained an additional 21.76 % of the total variance, was related to *p*-coumaryl acid isoprenyl ester (**Pa7**), caffeic acid prenyl ester (**Pa9**), kaemferide (**Fnl5**) and pinostrobin (**Fnn3**).

The principal component score plot and correlation scatterplot of the variables with PC1 and PC2 based on individual phenolic compounds are presented in Fig. 4. As seen in the PCA graph, the samples collected along the course of the Vardar River (MK5, 9, 10, 11, 12) are differentiated into a group in the negative part of PC1.

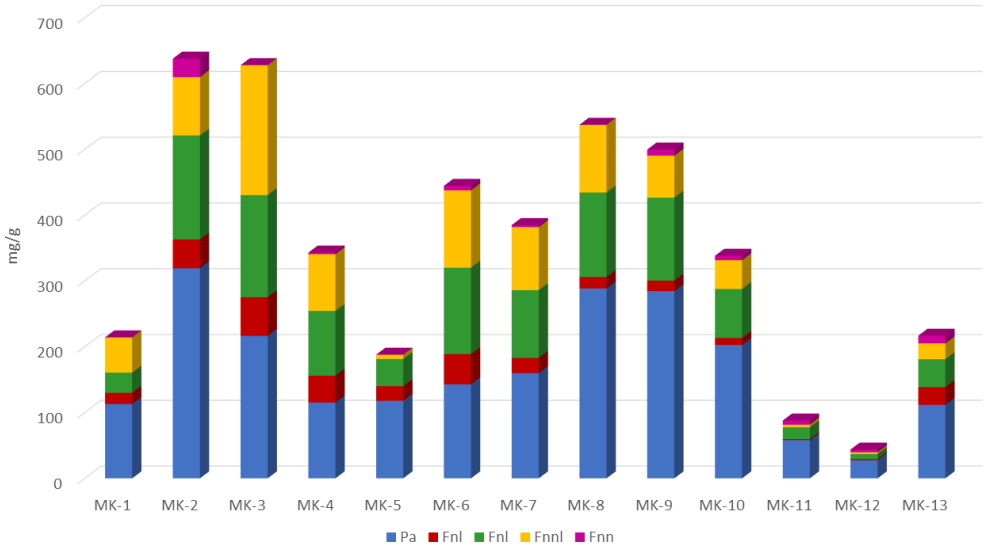
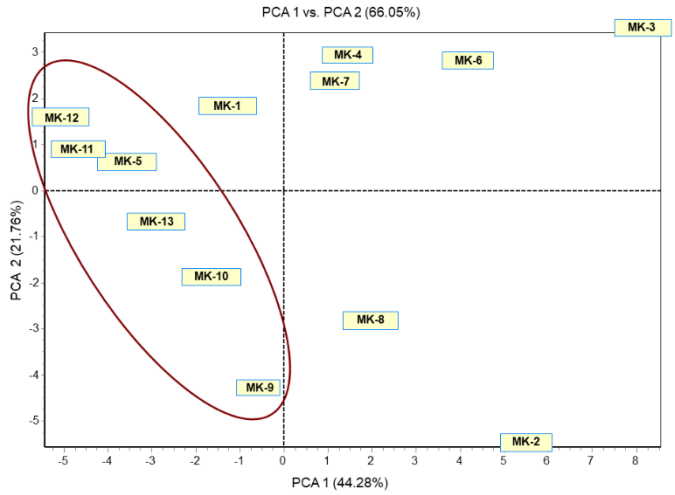
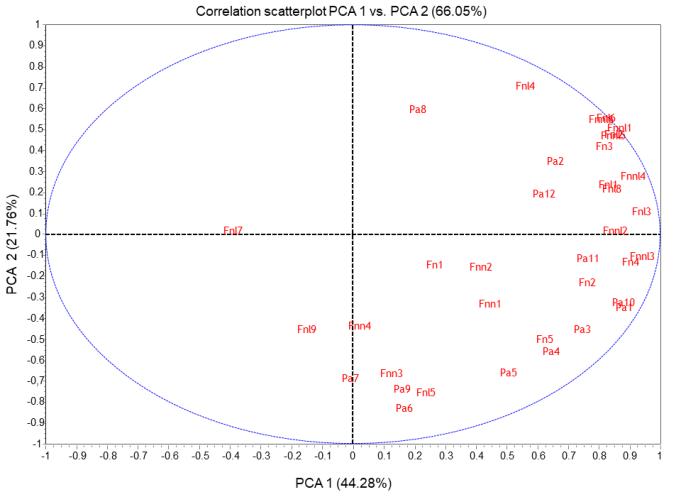


Fig. 3. Total content (mg/g) of each phenolic group: phenolic acids (**Pa**), flavonols (**Fnl**), flavones (**Fnl**), flavanonols (**Fnnl**) and flavanones (**Fnn**)



(a)



(b)

Fig. 4. (a) Principal component analysis score plot and (b) correlation scatterplot of the variables with PC1 and PC2 based on HPLC quantitative data for the individual compounds in the analyzed samples. For sample and compounds codes see Tables 1 and 2.

T a b l e 3

Individual and total phenolic content (mg/g, n=3) in propolis samples

	MK-1	MK-2	MK-3	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13
Phenolic acids and derivatives													
Pa1	11.74±0.83	30.39±2.60	25.20±1.29	8.82±0.41	6.53±0.13	17.63±1.04	11.69±1.03	22.07±0.41	14.49±0.24	14.27±0.27	3.56±0.06	1.83±0.10	7.22±0.21
Pa2	0.30±0.02	1.09±0.18	13.89±0.71	0.29±0.05	0.48±0.06	0.54±0.13	0.37±0.07	1.06±0.08	0.74±0.03	--	0.27±0.02	0.18±0.02	0.63±0.06
Pa3	5.36±0.47	10.36±0.85	8.93±0.41	7.13±0.35	5.53±0.16	7.15±0.54	7.07±0.72	10.07±0.45	10.40±0.04	5.28±0.13	4.22±0.08	1.57±0.06	6.77±0.97
Pa4	11.78±1.04	56.69±4.09	32.05±1.71	5.88±0.08	21.91±1.31	12.28±1.17	11.94±1.72	34.46±0.58	20.34±0.15	22.30±0.15	2.04±0.07	1.31±0.06	5.11±0.67
Pa5	--	26.18±2.41	10.18±0.41	7.13±0.39	8.06±0.19	16.29±1.56	9.70±1.13	23.77±0.77	36.08±0.36	9.09±0.46	--	--	5.86±0.75
Pa6	2.22±0.30	7.30±0.84	--	1.27±0.13	--	1.85±0.15	--	4.96±0.12	9.98±0.09	3.28±0.07	2.05±0.05	0.83±0.05	1.99±0.27
Pa7	--	22.53±2.76	--	--	11.51±0.98	--	18.49±2.93	21.42±0.14	58.96±1.13	17.39±0.28	14.31±0.16	5.19±0.16	1.83±0.17
Pa8	14.35±0.78	--	--	28.75±2.54	--	27.01±1.80	37.64±3.74	--	--	--	--	--	--
Pa9	35.03±2.61	83.22±2.43	35.07±4.77	19.11±1.12	52.11±2.59	13.48±1.45	15.38±1.28	115.38±6.49	62.65±1.40	97.38±1.86	19.40±0.18	7.20±0.58	43.09±1.84
Pa10	22.42±1.50	73.53±2.09	80.91±8.66	33.24±2.03	10.46±0.67	38.75±3.09	39.11±2.90	51.71±0.31	63.21±2.63	31.75±5.84	11.20±0.22	8.65±0.20	37.01±5.93
Pa11	--	3.06±0.18	2.01±0.06	0.54±0.05	--	1.27±0.05	--	--	--	--	--	--	0.48±0.17
Pa12	9.87±0.83	4.97±0.82	8.44±1.69	3.08±0.14	1.36±0.08	6.69±0.62	8.61±0.67	3.73±0.15	7.75±0.21	2.01±0.09	1.04±0.02	0.73±0.04	1.85±0.81
Total	**113.06±0.76**	**319.31±1.25**	**216.66±2.80**	**115.24±0.86**	**117.95±0.84**	**142.95±0.91**	**160.00±1.19**	**288.63±1.96**	**284.61±0.85**	**202.74±1.81**	**58.08±0.07**	**27.48±0.18**	**111.84±1.69**
Flavonols													
Fnl1	3.32±0.16	6.31±0.28	8.40±0.24	7.78±0.45	--	9.17±0.68	--	4.59±0.21	1.46±0.02	1.15±0.16	--	--	--
Fnl2	2.50±0.21	5.33±0.22	11.02±0.46	6.43±0.35	0.86±0.02	9.08±0.69	5.24±0.63	--	2.12±0.03	1.58±0.06	0.26±0.01	--	1.06±0.16
Fnl3	4.12±0.29	10.25±0.56	12.76±0.59	8.32±0.54	0.48±0.06	5.53±0.45	7.06±0.66	6.98±0.09	3.32±0.23	2.40±0.06	--	0.67±0.04	0.95±0.10
Fnl4	0.68±0.06	0.63±0.02	5.13±0.17	4.97±0.42	2.59±0.10	7.71±1.77	4.34±0.50	--	0.17±0.01	--	--	--	0.40±0.05
Fnl5	2.40±0.17	7.69±0.17	--	3.53±0.20	1.42±0.04	2.20±0.15	3.91±0.31	5.74±0.09	5.59±0.04	3.70±0.09	0.65±0.01	0.63±0.05	4.62±0.80
Fnl6	--	1.83±0.06	7.38±1.43	2.99±0.14	--	4.41±0.36	2.47±0.11	--	--	--	--	--	--
Fnl7	--	--	--	--	16.53±1.20	--	--	--	--	--	--	0.44±0.03	19.73±3.51
Fnl8	3.86±0.53	11.76±0.61	14.01±0.76	6.73±0.39	--	7.83±0.67	--	--	--	--	--	--	--
Fnl9	--	--	--	--	0.21±0.37	--	--	--	3.30±0.10	1.72±0.03	0.77±0.02	--	--
Total	**16.88±0.16**	**43.79±0.23**	**58.70±0.46**	**40.75±0.14**	**22.10±0.46**	**45.92±0.52**	**23.02±0.23**	**17.31±0.07**	**15.96±0.08**	**10.54±0.05**	**1.68±0.01**	**1.74±0.01**	**26.75±1.48**
Flavones													
Fn1	--	--	1.93±0.16	--	--	0.61±0.07	0.69±0.22	9.35±0.19	--	--	--	--	--
Fn2	6.19±0.32	22.90±1.31	21.88±1.32	12.02±0.66	17.08±1.04	9.61±0.71	11.94±1.01	16.53±1.18	11.67±0.03	7.68±1.57	1.98±0.37	1.77±0.09	8.83±1.68
Fn3	--	1.42±0.05	8.56±0.35	1.90±0.12	--	2.13±0.40	2.20±0.07	2.28±0.06	--	--	--	--	--

Fn4	55.23±3.83	115.62±1.40	102.14±8.64	84.59±7.83	22.45±2.81	112.39±4.40	88.13±5.19	88.79±3.17	99.44±6.12	51.92±1.15	12.12±0.22	5.86±0.23	25.54±3.38
Fn5	--	18.17±1.55	20.70±1.97	--	1.86±0.12	6.54±0.65	--	11.52±0.11	14.99±0.12	14.95±0.30	3.74±0.04	--	8.18±1.51
Total	**30.71±2.48**	**158.11±0.70**	**155.20±3.52**	**98.51±4.31**	**41.39±1.37**	**131.28±1.78**	**102.96±2.41**	**128.47±1.33**	**126.10±3.49**	**74.55±0.65**	**17.83±0.17**	**7.63±0.09**	**42.55±1.03**
Flavanonols													
Fnnl1	6.35±0.53	7.23±0.17	28.61±1.75	10.74±0.67	0.63±0.06	14.00±1.38	13.62±1.37	6.91±0.13	3.22±0.02	0.77±0.10	--	0.36±0.03	0.51±0.05
Fnnl2	11.85±0.92	25.46±0.50	60.37±4.02	12.80±0.73	2.19±0.20	17.87±1.10	18.84±3.77	42.30±0.47	16.60±0.37	15.60±0.44	2.21±0.03	1.36±0.04	7.57±0.90
Fnnl3	12.33±0.84	30.08±1.35	32.21±1.55	18.43±1.10	3.70±0.19	23.68±2.20	20.84±1.18	29.01±0.32	15.08±0.16	16.01±0.62	1.83±0.08	1.06±0.05	7.32±1.44
Fnnl4	9.07±0.55	13.88±1.03	30.01±1.38	19.09±1.49	--	26.56±2.11	17.52±0.96	13.65±0.27	16.38±0.35	5.63±0.04	--	--	--
Fnnl5	9.75±0.59	7.77±0.05	28.67±0.82	12.29±0.69	--	18.39±1.77	15.63±0.33	7.38±0.26	7.88±0.09	3.40±0.04	--	--	8.62±0.20
Fnnl6	4.27±0.48	4.25±0.25	17.63±0.38	12.86±0.58	--	17.15±1.45	9.54±0.44	3.67±0.06	4.70±0.18	2.33±0.16	--	--	--
Total	**53.63±0.18**	**88.67±0.52**	**197.51±1.26**	**86.21±0.35**	**6.52±0.08**	**117.66±0.43**	**95.99±1.26**	**102.91±0.14**	**63.86±0.14**	**43.74±0.24**	**4.04±0.04**	**2.78±0.01**	**24.03±0.64**
Flavanones													
Fnn1	--	4.48±0.46	--	--	--	2.26±0.24	0.91±0.01	--	--	--	--	--	0.96±0.19
Fnn2	--	0.77±0.11	--	--	--	0.98±0.21	--	--	--	0.40±0.07	--	--	--
Fnn3	--	21.16±1.88	--	2.12±0.19	--	3.58±0.41	2.04±0.06	--	9.26±0.38	5.97±0.16	6.21±0.10	3.19±0.32	9.86±2.02
Fnn4	--	1.66±0.13	--	0.32±0.01	--	--	--	0.12±0.02	--	0.37±0.02	--	0.92±0.04	0.89±0.33
Total	--	**28.07±0.84**	--	**2.44±0.13**	--	**6.81±0.11**	**2.96±0.03**	**0.12±0.02**	**9.26±0.38**	**6.73±0.07**	**6.21±0.10**	**4.11±0.19**	**11.71±1.02**
Total	**245.00±1.09**	**637.94±0.38**	**628.08±1.40**	**343.15±1.78**	**187.96±0.55**	**444.62±0.64**	**384.92±0.95**	**537.44±0.89**	**499.78±1.43**	**338.3±0.74**	**87.86±0.06**	**43.75±0.09**	**216.89±0.41**

4. CONCLUSIONS

The comprehensive analysis of Macedonian propolis revealed a rich chemical composition comprising phenolic acids and flavonoids, contributing to its health benefits and potential applications across various industries.[26]

In total, 36 compounds were detected and quantified, with the highest diversity and content of phenolic acids like caffeic and coumaric acid, along with their methyl, (iso)phenyl, benzyl, phenylethyl, and cinnamyl esters contributing up to 60 % of the total phenolic content. The presence of quercetin, kaempferol, and their methyl ethers was also observed. Pinobanksin was present as an aglycon, as well as its acetate, propionate, butyrate, and pentanoate. Characteristic compounds for the poplar type of propolis, including chrysin, acacetin, apigenin, naringenin, and pinostrobin were detected. However, the typical flavone galangin was not found in the analyzed samples, suggesting a peculiar characteristic of Macedonian propolis.

The total content of phenolic compounds, ranging from 43.75 to 637.94 mg/g, was higher compared to previously published data for propolis from Europe. This finding indicates that the economic potential of our country is high, and continued research into the properties and applications of Macedonian propolis can unlock further economic potential, including innovation and product development in related industries. Establishing quality standards and regulations for Macedonian propolis can enhance consumer confidence, ensuring product safety and efficacy. This regulatory framework can also facilitate market access and export opportunities.

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