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Application of LC–MS/MS with ion mobility for chemical analysis of propolis extracts with antimicrobial potential

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Abstract: The objective of this study was to test four-dimensional LC-ESI-MS/ /MS chromatography in analysis of complex mixture such as ethanol extracts of different propolis samples. In total more than 1200 picks were identified and only for 185 literature conformation was found. The given data represent the result of tentative identification, and summarized results are given in the text. Comparing the samples, from different altitudes, 96 components were detected as characteristic in high altitude samples and 18 in samples collected at low altitudes. Antimicrobial activity of ethanol extracts of propolis (EEP) and propylene glycol extracts of propolis (PGEP) were carried out on *S. aureus*, *B. cereus*, *M. flavus*, *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium*, *E. coli* and *E. cloacae* bacterial strains and compared with broad-spectrum antibiotics, streptomycin and ampicillin. Anti-quorum sensing activity was performed on *P. aeruginosa* by testing the effect of representative propolis extracts on biofilm formation, twitching and motility activity and production of pyocyanin. We demonstrated that the majority of explored propolis extracts have greater or equal minimal inhibitory concentration and minimum bactericidal concentration values compared to antibiotics, independently of the solvent used for the extraction. The samples collected from the highest altitude emerged as least active antimicrobial agents but with the greatest potential as anti-quorum sensing agents.

Keywords: tandem mass spectrometry; parallel accumulation serial fragmentation ion mobility mass spectrometer; poplar propolis; quorum sensing; *P. aeruginosa*.

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INTRODUCTION

Propolis is a mixture of a plenty health beneficial components which manifest antibiotic, antipyretic, antiviral, *etc.* activities. It's a mixture of plant resins with bee's wax. These properties have been known for centuries and are still of interest for scientific researchers all over the world.[1] There are a lot of different types of propolis, typically classified on the basis of geographical origin which is closely related to the present flora. The poplar propolis is the most represented in Europe. Its chemical composition is not unique and depends on the altitude were bees are kept. At altitudes above 500 m the main constituents of propolis resin are phenolic acids and their acetyl glyceryl esters. At lower altitudes (below 400 m) flavonoids and their derivatives are present.[2]

The 70 % ethanol (EEP) and propylene glycol (PGEP) extracts of propolis are most commonly used as auxiliary medicinal products on the prevention of diseases of the respiratory tract, such as influenza and colds, as well as strengthening the immune system.[3,4] It has been reported that EEP shows strong antibacterial activities against gram positive, and gram negative bacteria such as *Bacillus subtilis*, *Escherichia coli*, *Rhodobacter sphaeroides*,[5] *Helicobacter pylori*,[6] *Streptococcus aureus*,[7] *Lactobacillus plantarum* and *Pseudomonas spp.*[8] For this reason, propolis extracts were in high demand during the CoV 19 pandemic, due to the presence of quercetin, a potent antiviral agent.[9] The development of methods for the analysis of complex mixtures, such as propolis extracts, has always been a great challenge. According to the literature data, propolis contains over 300 different organic compounds in a wide range of concentrations and polarities, from waxes to sugars and from mg/g to pg/g.[10] In the case of complex mixtures, two or more different techniques are combined in order to obtain a complete picture of the chemical composition of the analyzed sample. HPLC and HPLC–MS chromatographic techniques, as well as GC and GC–MS, HPTLC, NMR, *etc.*, were used as the most common techniques in the analysis of ethanol extracts of propolis.[11–15] However, HPLC–MS has been shown to be the most suitable for the analysis of polar and medium polar compounds which are responsible for the vast majority of the therapeutic effects of propolis.[16] Over time, mass spectrometry has advanced considerably and there are a huge number of ion sources variations, analyzers and detectors that can be used.

For complete screening of complex samples, such as proplis, mass spectrometers with an ESI ion source and hybrid analyzers (Q TOF or Orbitrap) are usually used[16] as they can measure the precise mass and perform fragmentation of the analyzed molecule, also allow reduction of the chromatographic time and increase the resolution. In this way we can get information about each component: retention time, precise mass and isotopic distributions on the basis of which the molecular formula is obtained, with the pattern of molecule fragmentation. Comparison of these data with spectral libraries and/or with standards of pure

compounds enables identification of a large number of constituents. Because of the huge difference in polarity and concentration of individual components, it is impossible to identify them using single analysis. It is necessary to make modifications, in the sample preparation procedure, with the aim of eliminating the main components, since their high abundance are making analysis of the trace components very difficult. It is necessary to concentrate the sample and/or inject larger volumes which is impossible if the main components are not somehow removed from the sample. In 2016 Bruker has introduced a new generation of mass spectrometers with an ion mobility analyzer of a unique design located behind the ion source and in front of the quadrupoles and time-of-flight (TOF). Trapped ion mobility spectrometry or TIMS instrument introduces a fourth dimension in the analysis of complex mixtures. The separation of molecules based on their ion mobility adds additional information during the molecules identification process. By installing additional segment of analyzing sections to their TIMS cell design enabled development of parallel accumulation serial fragmentation (PASEF®) acquisition method.[17,18]

Herein, for the first time, we present the results obtained using timsTOF spectrometer, with PASEF technology, for analysis of propolis ethanol extracts. Furthermore, we have compered antimicrobial properties of two type of poplar propolis samples (their ethanol and propylene glycol extracts) on selected Gram positive and Gran-negative bacteria.

EXPERIMENTAL

Ethanol (Meilab, Serbia) and propylene glycol (Meilab, Serbia) extracts of poplar propolis samples collected from different altitude in Serbia (Table I) were prepared using 5 mL of solvents and 0.5 g of sample.

The samples were kept in the dark for 72 h at room temperature (25 °C) and filtered through filter paper. The resin content calculation was based on wax residues after extraction with 70 % ethanol. Propolis ethanol extracts profiling has been done on Bruker Elute LC system with Bruker Intensity Solo 1.8 C18-2, 2.1 mm×100 mm. Elution system was H_20 with 0.1 % formic acid (A) and MeCN with 0.1 % formic acid (B). The flow rate was 0.4 mL/min, and gradient program: 0–1 min, 0–2 % B; 1–10 min, 2–100 % B; 10–12 min, 100 % B. MS spectra were measured on timsTOF Pro MS system with ESI positive and negative ionization mode, scan mode with auto MS/MS and PASEF in range of 20-2000 *m*/*z*, and internal calibration. For data processing and interpretation Bruker MetaboScope 4.0 software with T-ReX 4D (time aligned region complete extraction) algorithm was used.

Antibacterial activity

The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240) and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210) and *Enterobacter cloacae* (human isolate), were used. The antibacterial assay was carried out by a microdilution method.[19] The bacterial suspensions were adjusted with sterile saline to a concentration of 10^5 CFU/mL. Compounds were dissolved in 70 % EtOH and PG solution (100 mg/mL) and immediately

added in Tryptic Soy broth (TSB) medium (100 μL) with bacterial inoculum (10^4 CFU per well). The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (*MIC*). The *MIC* obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on reduction of INT (*p*-iodonitrotetrazolium violet; 2-(4-iodophenyl)-3-(4-nitrphenyl)-5-phenyltetrazolium chloride, Sigma) color and compared with positive control for each bacterial strains. The *MBC* were determined by serial sub-cultivation of 2 μL into microtitre plates containing 100 μL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the *MBC*, indicating 99.5 % killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted extracts) and the positive control. Streptomycin (Sigma P 7794) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline). Five percent 70 % EtOh and PG were used as a negative control.

TABLE I. Samples location, altitude and resin content calculated on wax residues after extraction with 70 % ethanol. Samples from high altitude are bolded

Sample	Sampling place	Altitude, m	Resins content, %
1	Varvarin	150	42.50
2	**Luke, Ivanjica**	**550**	**38.05**
3	Irig	200	57.46
4	**Vukanja, Kruševac**	**500**	**44.70**
5	Vranje	300	33.92
6	Novi Sad	150	66.01
7	Petrovac na Mlavi	300	42.78
8	Rušanj V	200	67.76
9	V. Šiljegovac, Kruševac	200	62.73
10	Belegiš, Stara Pazova	200	51.26
11	Kovin	100	38.60
12	Banatski Karlovac I	150	57.57
13	Banatski Karlovac II	200	75.76
14	Deliblatska peščara	200	47.28
15	**Prijepolje I**	**450**	**61.51**
16	**Prijepolje II**	**900**	**59.06**

RESULTS AND DISCUSSION

LC–MS/MS analysis

The chemical composition of six representative samples, three from low altitude (6, 9 and 12) and three from high altitude (4, 15 and 16), were analyzed using Brukers tims TOF Pro with PASEF LC–MS/MS system on MetaboScope 4.0 platform for the first time. T-ReX 4D algorithm allowed us to combine ions belonging to the same compound into one feature, *i.e.*, isotopes, charge states, adducts or fragments. This non-linear retention time alignment ensure data consistency when chromatographic shifts between LC–MS runs occurre. Also, soft-

ware facilitates confident compound identification based on accurate mass, isotope pattern match, collisional cross sections (CCS) and convenient MS/MS data handling. MetaboScape 4.0 automatically identifies a known compound and characterizes an unknown in details. A fully integrated online data base query in combination with in silico fragmentation[21] for structure assignment and fully automated MS/MS spectral library queries (*e.g.*, Bruker HMDB Metabolite Library and Bruker MetaboBASE® Personal Library) from generated data. Through the use of data-dependent acquisition (DDA)[20] the mass spectrometer constantly cycles through a full scan acquisition (survey scan) and *N* MS/MS scans to ensure a sufficient number of sampling points across a chromatographic peak. This allows high quality MS/MS spectra regardless to the analyte concentration. In Table II the results of LC–MS/MS analysis were summarized in different classes of compounds (for more data see Supplementary material to this paper). It was obvious that the applied LC–MS/MS system detected an incomparably higher number of signals than previously reported in the literature. More than 1200 picks were fully characterized, and tentative identification was performed. The results were compared with literature data to determine if compounds had been previously detected in propolis. Literature data were found for 185 compounds (see Supplementary material) and most of them are: flavonoids, chalcones, phenols, phenolic acids and derivatives as well as terpenes, benzene derivatives, coumarine, saccharides, carboxylic acid, fatty alcohols, aldehyde, acids and their derivatives. Above 1000 detected signals have been reported for the first time as components of EEP. Only 96 components were detected in samples collected from high altitude, while only18 components were detected in samples collected from low altitude.

Results of antimicrobial tests

Uncontrolled and frequent use of antibiotics led to the emergence of resistant strains of bacteria, especially in hospital settings such as methicillin-resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa*.[22] Great efforts have been made to solve this problem, by developing new antibiotics and/or testing known natural products. It seems that good candidates are different types of propolis and its extracts. A number of studies showed high efficiency of Brazilian propolis against staphylococci and other pathogenic microorganisms for humans and animals. The Regueira group reported high activity of Brazilian red propolis against *S. aureus*, *E. coli* and *P. aeruginosa*.[23] The Wojtyczka and coworkers published the results on the effect of ethanoic extract of Polish propolis (EEPP) against methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) clinical isolates.[24] However, the authors used the propolis from only one location-Kamianna (South Poland). The same group of authors also proved the data of high activity of Polish propolis against biofilm forming *S. epidermidis* strains,[25] and

TABLE II. LC–MS/MS tentative identification of different classes of compounds in propolis samples

Compounds classes	Total number of compounds	
	Detected	Previously reported
Flavonoids and chalcones	182	53
Fatty alcohols, aldehydes, acids and their derivatives	158	11
Phenols, phenolic acids and derivatives	111	50
Carboxylic acids	108	9
Terpenes	88	25
Esters	55	
Benzene derivatives	54	16
Vitamins B and D and their derivatives	31	
Carboxamide	17	
Pyran and furan derivatives	16	2
Alkene and polyene	15	
Coumarine	15	9
Amines	14	1
Prostaglandins	14	
Carbohydrates	9	9
Lactones	8	

what is from a clinical point of view, probably the most beneficial aspect of propolis. The biofilm form of staphylococci has slightly lower susceptibility then planktonic cells with much lower differences compared to other antimicrobial agents, *e.g.*, antibiotics.

In this study, sixteen propolis samples, from different regions of Serbia, were collected and ethanol and propylene glycol extracts prepared. The antibacterial activity of all extracts was verified using four G+ bacterial strains *S. aureus*, *B. cereus*, *M. flavus* and *L. monocytogenes*, and four G– bacterial strains *P. aeruginosa*, *S. typhimurium*, *E. coli* and *E. cloacae.* The minimum inhibitory (*MIC*) and minimum bactericidal concentration (*MBC*) were determined and compared with two controls, broad-spectrum antibiotics streptomycin (protein synthesis inhibitor) and ampicillin (*β*-lactam antibiotic). The results of *MIC* and *MIB* are given in Tables III–VI. All tested bacterial stream had lower *MIC* and *MIB* values for streptomycin than ampicillin. Therefore, *MIC* and *MIB* values of tested extracts, lower or equal to streptomycin, were considered more active than the control. Values higher or equal to ampicillin, were considered less active than control. Values between these two limit values can be considered equivalent to activity of antibiotics. *MIC* and *MBC* for tested EEP and PGEP were different depending on the sample and bacterial culture (Tables III and V). For *E. cloacae* and *S. typhimurium* EEP and PGEP have greater or equal activity in comparison to control antibiotics except for sample 16. PGEP have greater or equal activity for *S. aureus* while EEP have greater or equal activity for *B. cereus.* Both extracts showed the

equal or lower activity compared to the controls for *L. monocytogenes* and *M. flavus*. Both extracts have shown almost the same activity for *E. cloacae* as the antibiotics. Other bacterial cultures (*P. aeruginosa* and *E. coli*) are equally, less or more sensitive to propolis extracts as the antibiotics, depending on the tested sample and used extracts. If we compare different samples and the altitude of collections, there is lower sensitivity of the tested bacterial cultures to propolis samples collected at higher altitudes than at lower ones (Tables III and IV).

TABLE III. Antibacterial activity of EEP (*MIC* in mg/mL). *S.a.* – *Staphylococcus aureus*; *B.c.* – *Bacillus cereus*; *M.f.* – *Micrococcus flavus*; *L.m.* – *Listeria monocytogenes*; *P.a.* – *Pseudomonas aeruginosa*; *E.c.* – *Escherichia coli*; *En.cl.* – *Enterobacter cloacae*; *S.t.* – *Salmonella typhimurium*

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
1	0.25	0.25	0.3	0.25	0.06	1.25	0.125	0.25
2	0.02	0.3	0.125	0.75	0.2	0.5	0.75	0.125
3	0.5	0.125	0.9	0.9	0.6	0.9	0.9	0.7
4	0.3	0.25	1.25	0.25	0.25	0.3	0.25	0.06
5	0.03	0.06	0.06	0.25	0.125	0.6	0.7	0.125
6	0.125	0.06	0.03	0.125	0.03	0.25	0.25	0.03
7	0.03	0.03	0.6	0.5	0.06	0.6	0.6	0.05
8	0.4	0.2	0.4	0.4	0.4	0.4	0.4	0.125
9	0.2	0.125	0.5	0.25	0.2	0.5	0.2	0.125
10	0.2	0.2	0.2	0.25	0.06	0.4	0.125	0.2
11	0.02	0.3	0.2	0.3	0.04	0.3	0.5	0.25
12	0.2	0.25	0.3	0.5	0.3	0.3	0.5	0.2
13	0.125	0.125	0.125	0.6	0.5	0.25	0.25	0.25
14	0.06	0.06	0.06	0.125	0.25	0.125	0.25	0.06
15	0.4	0.4	0.8	0.25	0.8	0.5	0.25	0.4
16	0.8	0.5	0.5	1	0.5	0.5	0.25	0.5
Streptomycin	0.1	0.2	0.05	0.2	0.2	0.3	0.2	0.2
Ampicillin	0.3	0.3	0.3	0.4	0.3	0.4	0.8	0.3

TABLE IV. Antibacterial activity of EEP (*MBC* in mg/mL). *S.a.* – *Staphylococcus aureus*; *B.c.* – *Bacillus cereus*; *M.f.* – *Micrococcus flavus*; *L.m.* – *Listeria monocytogenes*; *P.a.* – *Pseudomonas aeruginosa*; *E.c.* – *Escherichia coli*; *En.cl.* – *Enterobacter cloacae*; *S.t.* – *Salmonella typhimurium*

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
1	0.5	0.5	1	0.5	0.125	2.5	0.25	0.5
2	0.03	0.5	0.5	1	0.25	1	1	0.5
3	1	0.25	1.25	1.25	1.25	1.25	1.25	1
4	0.5	0.5	2.5	1	0.5	0.5	2.5	0.125
5	0.06	0.125	0.125	0.5	0.25	1.25	1	0.25
6	0.25	0.125	0.06	0.25	0.06	0.5	0.5	0.06

TABLE IV. Continued

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
7	0.06	0.06	1.25	1	0.125	1.25	1.25	0.06
8	0.5	0.25	0.5	0.5	0.5	0.5	0.5	0.25
9	0.25	0.25	1	0.5	0.25	1	0.25	0.25
10	0.25	0.25	0.25	0.5	0.125	0.5	0.25	0.25
11	0.03	0.5	0.25	0.5	0.06	0.5	1	0.5
12	0.25	1	0.5	1	0.5	0.5	1	0.25
13	0.25	0.25	2.5	1.25	1	0.5	0.5	0.5
14	0.125	0.125	0.3	0.25	0.5	0.25	0.5	0.125
15	0.5	0.5	1	0.5	1	1	0.5	0.5
16	1	1	1	6	1	1	0.5	1
Streptomycin	0.2	0.3	0.1	0.3	0.3	0.5	0.3	0.3
Ampicillin	0.4	0.4	0.4	0.5	0.5	0.8	1.25	0.5

The results for MBC are similar to those for MIC, *S. aureus*, *P. aeruginosa* and *S. typhimurium* were the most sensitive, and *M. flavus* and *L. monocytogenes* the least sensitive to propolis extracts. Ethanol and propylene glycol extracts of propolis showed no obvious difference in their activity, probably due to the similar polarity of used solvents.[26]

EEPs produced from samples collected in different geographical regions, *e.g.*, Albania, Turkey,[27] Russia[28] and Brazil have been observed in other studies.[29] The virulence-related factors of *P. aeruginosa*, such as twitching and motility activity, biofilm formation and pyocyanin production were tested for ethanol and propylene glycol extracts of four representative samples (two low and two high altitudes). Results are presented in Tables VII and VIII and Figs. 1 and 2. Based on the data shown , sample **16** from the highest altitude, inhibits biofilm formation at low concentrations, regardless to the type of extract. Only ethanol extract of sample **5** showed strong activity, better than streptomycin and similar to ampicillin Table VII.

TABLE V. Antibacterial activity of PGEP (*MIC* in mg/mL). *S.a. – Staphylococcus aureus*; *B.c. – Bacillus cereus*; *M.f. – Micrococcus flavus*; *L.m. – Listeria monocytogenes*; *P.a. – Pseudomonas aeruginosa*; *E.c. – Escherichia coli*; *En.cl. – Enterobacter cloacae*; *S.t. – Salmonella typhimurium*

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
1	0.125	0.25	0.25	0.5	0.5	0.75	0.125	0.25
2	0.02	0.5	0.25	0.75	0.06	0.06	0.75	0.015
3	0.25	0.5	0.5	0.125	0.5	0.125	0.6	0.004
4	0.6	0.6	0.2	1.25	0.25	1.25	0.25	0.6
5	0.25	0.25	0.3	0.06	0.03	0.25	0.5	0.125
6	0.125	1.25	1.25	2.5	2.5	1.25	1.25	0.5
7	0.125	0.25	1.25	0.5	0.25	0.25	0.25	0.25

TABLE V. Continued

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
8	0.004	0.5	0.2	0.5	0.015	0.3	0.25	0.008
9	0.025	0.2	0.2	0.2	0.2	0.125	0.2	0.008
10	0.125	0.2	0.2	0.2	0.2	0.25	0.06	0.125
11	0.004	0.2	0.008	0.25	0.012	0.125	0.25	0.006
12	0.125	0.2	0.2	0.25	0.06	0.2	0.125	0.02
13	0.03	0.03	0.03	1.7	0.6	1.7	1.25	0.6
14	0.006	0.3	0.25	0.3	0.2	0.2	0.3	0.008
15	0.012	0.25	0.3	0.75	0.2	0.3	0.5	0.012
16	0.5	1.5	0.3	1	0.25	2	2	1
Streptomycin	0.1	0.2	0.05	0.2	0.2	0.3	0.2	0.2
Ampicillin	0.3	0.3	0.3	0.4	0.3	0.4	0.8	0.3

TABLE VI. Antibacterial activity of PGEP (*MBC* in mg/mL). *S.a.* – *Staphylococcus aureus*; *B.c.* – *Bacillus cereus*; *M.f.* – *Micrococcus flavus*; *L.m.* – *Listeria monocytogenes*; *P.a.* – *Pseudomonas aeruginosa*; *E.c.* – *Escherichia coli*; *En.cl.* – *Enterobacter cloacae*; *S.t.* – *Salmonella typhimurium*

Compound	Bacterium							
	S.a.	*B.c.*	*M.f.*	*L.m.*	*P.a.*	*E.c.*	*En.cl.*	*S.t.*
1	0.25	0.5	0.5	1	2.5	1	0.25	0.5
2	0.03	1	0.5	1	0.125	0.125	1	0.03
3	0.5	1	1.25	1.25	1	0.25	1.25	0.008
4	0.25	0.25	0.25	2.5	0.5	2.5	1.25	1.25
5	0.5	0.5	0.5	0.125	0.125	0.5	1	0.25
6	0.25	2.5	2.5	5	5	2.5	2.5	1
7	0.25	0.5	2.5	1	0.5	0.5	0.5	0.5
8	0.008	1	0.25	1	0.03	0.5	0.5	0.015
9	0.03	0.25	0.25	0.25	0.25	0.25	0.25	0.016
10	0.25	0.25	0.25	0.25	0.25	0.5	0.125	0.25
11	0.008	0.25	0.015	0.5	0.015	0.5	0.5	0.008
12	0.25	0.25	0.25	0.5	0.125	0.25	0.25	0.03
13	0.06	0.06	0.06	2.5	1.25	2.5	2.5	1.25
14	0.008	0.5	0.5	0.5	0.25	0.25	0.25	0.15
15	0.016	0.5	0.5	1	0.25	0.5	1	0.016
16	1	2	0.5	6	0.5	4	4	2
Streptomycin	0.2	0.3	0.1	0.3	0.3	0.5	0.3	0.3
Ampicillin	0.4	0.4	0.4	0.5	0.5	0.8	1.25	0.5

On the other hand, sample **2** completely reduced colony edge protrusions, regardless to the solvent used for extraction. The same sample reduce pyocyanin production for 45 % when prepared as PGEP at the similar level as used controls. Samples **5E**, **5PG**, **8E** and **16E** were less effective, decreasing pyocyanin production for 70, 90, 85 and 75 % respectively.

TABLE VII. Effects of propolis (**2E**, **2PG**; 5E, 5PG; 8E, 8PG; **16E** and **16PG**) on biofilm formation of *P. aeruginosa* (PAO1); biofilm formation values were calculated as: (mean A_{620} treated well/mean A_{620} control well)×100. Values are expressed as means ± *SE*

Agent	Biofilm formation, %		
	0.5*MIC*	0.25*MIC*	0.125*MIC*
5E	83.55±4.73	–	–
5PG	91.43±1.50	91.56±0.97	89.92±0.64
8E	83.94±0.19	88.31±1.83	42.02±0.51
8PG	88.27±2.18	92.29±4.73	93.52±2.06
2E	**94.63±0.51**	**95.23±2.96**	**77.19±1.73**
2PG	**91.20±2.37**	**91.88±0.94**	**91.77±3.76**
16E	**82.47±1.48**	**15.35±0.51**	**–**
16PG	**77.85±1.26**	**8.65±0.65**	**–**
Ampicilin	14.88±1.14	–	–
Streptomicin	86.78±41.72	36.36±1.90	29.42±0.94

TABLE VIII. Twitching and motility activity of representative propolis samples

Agent	Colony diameter ± *SD*, mm	Colony color	Protrusions diameter, μm	Colony edge on microscope
5E	7.33±0.58	White	40-136	Slightly reduced protrusion
5PG	8.00±2.08	White	40-80	Partly reduced protrusion
8E	6.33±0.58	White	24-64	Partly reduced protrusion
8PG	9.33±1.53	Light green	24-80	Partly reduced protrusion
2E	**18.67±4.73**	**Light green**	**-**	**Reduced protrusion**
2PG	**10.33±1.53**	**White**	**-**	**Reduced protrusion**
16E	**6.67±0.58**	**White**	**8-40**	**Partly reduced protrusion**
16PG	**10.33±0.53**	**Light green**	**40-120**	**Partly reduced protrusion**
Streptomycin	7.67±0.58	White	40-120	Slightly reduced protrusion
Ampicillin	8.00±0.00	White	64-160	Slightly reduced protrusion
Control PAO1	21.0±3.60	Green	80-240	Regular protrusion

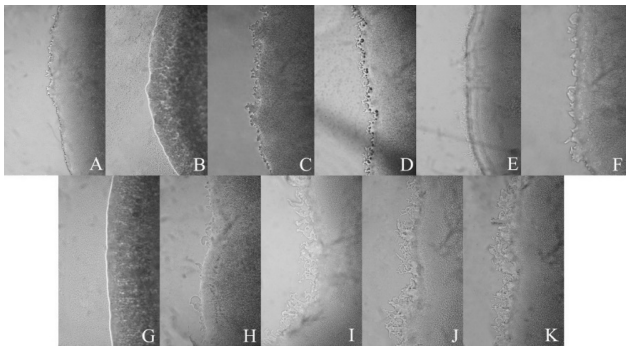

Fig. 1. Light microscopy of colony edges of *P. aeruginosa* in twitching motility, grown in the presence or absence of propolis at a concentration of 0.5 MIC. The colonies from the bacteria grown with: **2E** (A); **2PG** (B); 5E (C); 5PG (D); 8E (E); 8PG (F); **16E** (G); **16P**G (H); streptomycin (I); ampicillin (J); *P. aeruginosa*, control (K); magnification: (A–E)×100.

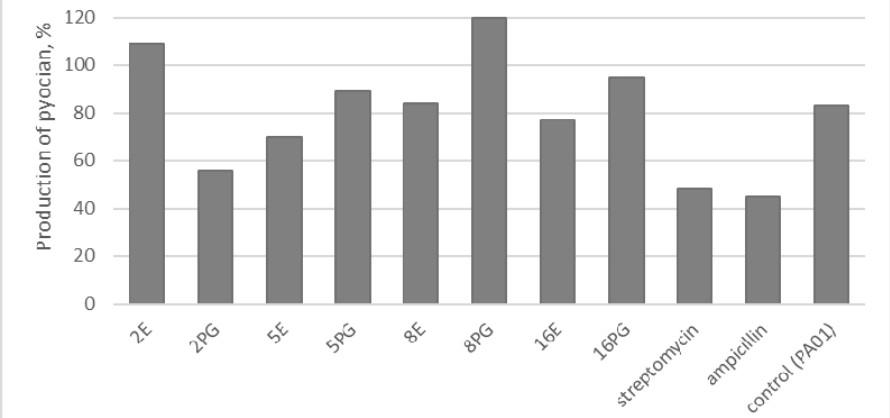

Fig. 2. Effects of propolis extracts (**2E**, **2PG**; 5E, 5PG; 8E, 8PG; **16E** and **16PG**) at 0.5*MIC* on pyocyanin production by *P. aeruginosa* (PAO1). The pyocyanin production was estimated by measuring optical density on 520 nm (OD_{520}) and 600 nm (OD_{600}) and calculated by formula: $I = 100(OD_{520}/OD_{600})$.

CONCLUSION

This paper presents the results of four-dimensional LC–ESI-MS/MS chromatography analysis of propolis samples collected on different locations and altitudes. More than 1200 picks were tentatively identified and for only 185 literature conformation have been found. This indicates the great potential of this technique. The 96 components were obtained only in samples collected from high altitude, while 18 components were obtained only in samples collected from low altitude. Antimicrobial activity of EEP and PGEP extracts for some of the most persistent human Gram-positive and Gram-negative bacteria was equal and/or better compared to broad-spectrum antibiotics used as the control. Comparison of the results, of antimicrobial activity with the collection altitude of the propolis samples, indicate that the sample collected on the highest altitude has the largest anti- quorum sensing potential.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11064, or from the corresponding author on request.

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ИЗВОД

ПРИМЕНА LC–MS/MS ТЕХНИКЕ СА ЈОНСКОМ ПОКРЕТЉИВОШЋУ ЗА ХЕМИЈСКУ АНАЛИЗУ ЕКСТРАКАТА ПРОПОЛИСА СА АНТИМИКРОБНИМ ПОТЕНЦИЈАЛОМ

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Циљ овог истраживања је било тестирање четвородимензионалне LC–ESI-MS/MS хроматографије у анализи комплексних смеша, као што су етанолни екстракти различитих узорака прополиса. Укупно је идентификовано више од 1200 пикова, а само за 185 смо нашли литературну потврду. Приказани подаци представљају тентативну идентификацију и сумирани резултат је дат у тексту. Поређењем узорака са различитих надморских висина, за 96 једињења је утврђено да се налазе само у узорцима са високе надморске висине и 18 само у узорцима прикупљеним на нижим надморским висинама. Антибактеријске активности етанолних и пропиленгликолних екстрактата прополиса (EEP и PGEP, редом) тестиране су на *S. aureus*, *B. cereus*, *M. flavus*, *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium*, *E. coli* и *E. cloacae* бактеријским линијама и поређене са антибиотицима широког спектра деловања, стрептомицином и ампицилином. *Anti-quorum sensing* активност је тестирана на *P. aeruginosa* испитивањем ефекта репрезентативних екстраката прополиса на формирање биофилма, тестовима покретљивости руба колоније (*twitching* и *mobility*) и производње пиоцианина. Показали смо да највећи број, коришћених екстраката прополиса, има исте и/или мање *MIC* и *MBC* вредности, у поређењу са атибиотицима, независно од растварача коришћеног за екстракцију. Узорци прикупљени на високим надморским висинама су се показали као најмање активни антибактерициди али имају велики *anti-quorum sensing* потенцијал.

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