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Original article

Activity of selected plant extracts against honey bee pathogen *Paenibacillus larvae*

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Abstract – The present study focuses on the antibacterial activity of selected plant extracts against *Paenibacillus larvae*, the causal agent of American foulbrood disease of honey bees. A gas chromatography-mass spectrometry method was used to analyze six diethyl ether extracts of two white birch species, black poplar and common aspen buds, as well as *n*-hexane, diethyl ether, and methanol extracts of young twigs from downy and silver birches. Among the identified extract constituents were flavonoids, phenylpropenoids, triterpenoids, and glucosides. In spite of significant differences in qualitative and quantitative composition, all tested in vitro extracts demonstrated high anti-*P. larvae* activity at minimal inhibitory concentration levels between < 1.0 and 125 μg/mL. To examine the cause of such disparity, the anti-*P. larvae* activity of some individual constituents naturally present in plant extract compounds was determined. A higher susceptibility of *P. larvae* (ERIC I) to relatively poorly polar triterpenoid levels compared to polar compounds, flavonoids, and glucosides was demonstrated.

honey bees / *Paenibacillus larvae* / plant extracts / antimicrobial activity

1. INTRODUCTION

Serious problems in worldwide beekeeping are connected to American foulbrood (AFB), a honey bee larvae disease caused by the Gram-positive spore-forming bacterium *Paenibacillus larvae.* The pathogen produces spores which are extremely environmentally resistant. Young larvae are infected by ingesting spores and they will die when sporulation occurs. This global disease is one of the primary honey bee threats (Genersch 2010; Evans and Schwartz 2011). The results of a 5-year epidemiological investigations show the scale of the threat in Poland: from 4090 inspected apiaries, 38% were infected (Skubida et al. 2014). For a long time, apiculturists treated the sick colonies with antibiotics. However, antibiotic treatment leads to negative consequences, such as appearance of bacterial resistance (Miyagi et al. 2000; Gende et al. 2010) and shifts in the gut microbial community structure which have detrimental impact on honey bees (Raymann et al. 2017). Current European legislation does not allow to use antibiotics for treatment of honey bees (Mutinelli 2003), because the products of these "farming animals" cannot contain drug residue. This prohibition has greatly limited the possibility of the treatment, but at the same time stimulate the development of alternative methods of combating AFB. Replacement of synthetic chemical drugs with natural antimicrobial substances is one promising direction (Alonso-Salces et al. 2017).

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As a natural remedy essential oils of different plants were examined. The antimicrobial action of essential oils can be mostly attributed to monoterpene hydrocarbons and their oxygenated derivatives, monoterpenoids. Anti-*P. larvae* activity has already been tested with the use of different citrus oils, thyme, oregano, rosemary, eucalyptus, and others (Alippi et al. 1996; Albo et al. 2003; Chaimanee et al. 2017; Fuselli et al. 2006, 2008; Gende et al. 2009; González and Marioli 2010; Pellegrini et al. 2017a, b; Santos et al. 2012). Unfortunately, the application of essential oils has not lead to the desired effect: the majority of them demonstrated relatively low activity against *P. larvae* with minimal inhibitory concentration (MIC) values from 250 to 850 μg/mL (Alonso-Salces et al. 2017). According to Albo et al. (2003), who evaluated the effectiveness of essential oils from savory, thyme, lemongrass, and oregano, neither single essential oils nor blends were effective in the elimination of AFB clinical symptoms at any dose formulation. Moreover, colonies treated with these oils showed higher levels of infection than those receiving a control treatment (Albo et al. 2003).

Substantially, more active against the pathogen is propolis (Antúnez et al. 2008; Bastos et al. 2008; Bilikova et al. 2013; Chen et al. 2018; Isidorov et al. 2016, 2017; Mihai et al. 2012; Wilson et al. 2015; Simone-Finstrom et al. 2017). Extracts of this natural antimicrobial bee product demonstrated lower (as a rule, an order of magnitude) MIC values in tests with *P. larvae* than essential oils. However, propolis is a highly valued bee product and demand for it is still growing. Moreover, it plays an extremely important role in bees "social immunity" (Borba et al. 2015; Simone-Finstrom and Spivak 2010). Hence, the excessive "robbery" of hives with this natural antibiotic responsible for the lower bacterial load levels within the hive is highly undesirable. The antibacterial activity of propolis has been reported to be primarily due to phenolics such as flavonoids, hydroxycinnamic, and phenolcarboxylic acids and their esters (Burdock 1998; Kujumgiev et al. 1999; Popova et al. 2007, 2017). However, these compounds are the secondary metabolite of plants and some recent investigations showed the anti-AFB potency of plant extracts (Boligon et al. 2013; Flesar et al. 2010; Reyes et al. 2013). In this respect, there is special interest in plants from which honey bee collect resins to prepare propolis. In the boreal and temperate climate zones of Europe, the principal plant precursors of propolis are bud resins of black poplar (*Populus nigra*) and its cultivars, white birch (*Betula pubescens*), and common aspen (*Populus tremula*) (Bankova et al. 2006; Isidorov et al. 2014a, 2016).

The main goal of this work was to investigate the in vitro anti-*P. larvae* activity, expressed as diameter of inhibition zone and as MIC value, of different extracts from some parts of the abovementioned "resin-giving" plants and attempt to connect the observed effects with the chemical composition of the extracts.

2. MATERIALS AND METHODS

2.1. Chemicals and plant material

Chemicals Betulinic, ursolic, and oleanolic acids, lupeol, dipterocarpol, betulinol, quercetin, (+)-catechin, salicin, arbutin, rhaponticin, and baicalin, and pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA), as well as C_8-C_{40} *n*-alkane calibration standards, were purchased from Sigma-Aldrich (Poznań, Poland). Extractions were carried out with *n*-hexane, diethyl ether, and methanol (POCH SA, Gliwice, Poland). Diethyl ether was distilled before use.

Plant material Buds of downy birch (*B. pubescens*), silver birch (*B. pendula*), common aspen (*P. tremula*), and black poplar (*P. nigra*) were gathered in April and/or September 2015 in the forests around Bialystok, Poland (53° 08′ 07″ N; 23° 08′ 44″ E). Buds were collected from 20- to 35-year-old trees (from 5 to 6 trees of each taxon). Besides that, plant material included twigs with young leaves from both species of birch collected in April 2015. In order to identify birch species, the genomic DNA was extracted from fragments of birch leaves and alcohol dehydrogenase was used to study the nuclear DNA sequences (Isidorov et al. 2014a).

Sample preparation and chemical analysis Buds (2–5 g) were cooled overnight at – 18 °C, ground

and transferred into a 100-mL retort, and extracted by stirring with three 50-mL portions of diethyl ether for 30 min. The joint extracts were filtered through a paper filter and then the ether was removed using a rotor evaporator to obtain the brownish residue which were used for chemical analysis and antibacterial tests. For chemical analysis, about 5 mg of the residue was diluted with 220 μL of pyridine and 80 μL of BSTFA was added. The mixture was sealed and heated for 30 min at 60 °C to form trimethylsilyl (TMS) derivatives.

Young birch twigs from last year's accretion (ca. 5–6 g) were air-dried, cut into small pieces, ground, and extracted successively with three 100-mL portions of *n*-hexane, diethyl ether, and methanol. Next, the joint extracts were filtered and the solvent was evaporated to dryness. TMS derivatives were prepared from the obtained residue of the extracts using the technique described above.

The obtained solutions were analyzed by a GC-MS method on a HP 7890 gas chromatograph with the 5975 VL MSD Triple-Axis Detector (Agilent Technologies, USA). The apparatus was fitted with an HP-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness), with electronic pressure control and split/splitless injector. The latter worked at 250 °C in the split (1:50) mode. The helium flow rate through the column was 1 mL/min. The initial column temperature was 50 °C, rising to 320 °C at 3 °C/min, and the higher temperature was maintained for 15 min. The MSD detector acquisition parameters were as follows: the transfer line temperature was 280 °C, the MS source temperature 230 °C, and the MS quad temperature 150 °C. The electron impact mass spectra were obtained at 70 eV of ionization energy. After integration, the fraction of separated components in the total ion current (TIC) was calculated.

To identify the separated components, both mass spectral data and calculated linear temperature-programmed retention indices (I^T) were used. Mass spectrometric identification was carried out with an automatic system of GC-MS data processing supplied by NIST and home-made mass spectra libraries. The latter contains more than 1800 spectra of TMS derivatives prepared from commercial preparations of flavonoids, other phenolics, terpenoids, aliphatic acids, alcohols, and carbohydrates.

Retention indices were calculated from the results of the separation C_8–C_{40} *n*-alkane solutions in hexane and the TMS derivatives. The obtained I^T values were compared with NIST collection (NIST 2013) as well as with the authors' previously published data (Isidorov et al. 2009, 2014a, b; Isidorov 2015). Identification was considered reliable if the results of a computer search in the mass spectra library were confirmed by the experimental I^T values, i.e., if their deviation from the averaged literature values did not exceed ± 10 u. i. (inter-laboratorial deviation for low polar stationary phases).

2.2. *P. larvae* strain isolation

P. larvae were isolated from dead honey bee larvae and honey sample originating from apiaries in south-eastern Poland with AFB-diseased colonies (Isidorov et al. 2017). Briefly, dead larvae were aseptically removed from brood combs and then were crushed and suspended in 5 mL of physiological saline (0.9% NaCl), and shook for 10 min. To isolate bacteria from honey samples, 10 mL of honey was preheated to 45 °C, diluted with sterile water (a ratio of 1:1), and centrifuged at 3000×*g* for 30 min. The resulting pellets were suspended in 200 μL of 0.9% NaCl. The suspensions of crashed larvae and honey were centrifuged at 10,000 rpm for 5 min, and the resulting pellets were suspended in 900 μL of 0.9% NaCl. All samples were preheated for 10 min at 85 °C in order to eliminate vegetative cells and to select the endospores, spread on a Columbia Blood Agar Base supplemented with vitamin B_1 (Oxoid, Basingstoke, England), and incubated at 30 °C in 5% of CO_2 for 48–96 h. Those Gram-positive bacteria forming round, transparent, or slightly whitish colonies with a diameter of about 2–3 mm, catalase-negative, and creating spiral forms in the Plagemann probe were classified as *P. larvae* (Neuendorf et al. 2004; Plagemann 1985). The *P. larvae* strain LMG 09820 (Belgian Coordinated Collections of Microorganisms) was used as a control.

2.3. *P. larvae* strain ERIC genotyping

According to Genersch et al. (2006), four ERIC (enterobacterial repetitive intergenic consensus) genotypes, ERIC I–IV, could be identified among *P. larvae* strains. However, only ERIC I and II are the practically important genotypes while ERIC III and IV have not been registered in field isolates and exist only in culture collections (Alippi et al. 2004; Antúnez et al. 2007; Genersch and Otten 2003). For ERIC genotyping, total DNA of the "wild" *P. larvae* strains under study was prepared from the 48-h cultures of the isolates grown in Mueller-Hinton broth (Oxoid) using a DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) and the QIAcube apparatus (Qiagen) according to the manuals for Gram-positive bacteria. The DNA level and purity were checked using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific Com., Waltham, USA).

ERIC genotyping was performed using primers designed previously (Versalovic et al. 1994). The PCR reactions were carried out with 1.5 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 250 ng of DNA, 0.5 mM of each of the four dNTP, 1.5 mM $MgCl_2$, and 1.0 μM of each primer in a total volume of 25 μL. The PCR conditions were as follows: a single denaturation step at 94 °C for 5 min, 35 cycles of denaturation of the DNA template at 95 °C for 1 min, annealing of primers at 57 °C for 1 min, and extension of PCR products at 72 °C for 2.5 min. An extra extension step was performed at 72 °C for 10 min. Amplified products and two DNA markers, GeneRuler DNA Ladders 100 bp and GeneRuler DNA Laders 1 kb Thermo Fisher Scientific Co., were separated in a 1.2% agarose gel and stained with ethidium bromide (0.5 μg/mL). The gels were photographed under UV light using the ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, USA). The PCR was prepared in triplicate.

2.4. Antibacterial activity of plant extracts

The prepared plant-derived extracts and selected chemicals (pure triterpenoids, flavonoids, and glucosides) were tested against wild *P. larvae* isolates, as well as against the reference strain LMG 09820. The plant extracts and pure chemicals were dissolved in DMSO at concentration, respectively, of 10 mg/mL and 100 mM, sonicated in the Branson 2510 ultrasonic bath (Sigma), and filtrated with a 0.22-μm pore size Rotilabo-Spritzenfilter filter (Carl Roth GmbH and Co, Karlsruhe, Germany). The bacterial strains kept at – 80 °C were inoculated onto blood agar (Oxoid) and incubated for 48 h at 37 °C. Then, bacteria were re-inoculated into Mueller-Hinton broth and incubated until the cultures reached an optical density of 0.2–0.3 at 600 nm, measured with a V-670 spectrophotometer (Jasco, Japan).

Firstly, the antimicrobial activity of the plant extracts against *P. larvae* isolates and the reference strain was investigated by the agar well diffusion test. Shortly, an overnight culture (100 μL) of microorganism of OD_{600}~0.2 was carefully and evenly spread over Mueller-Hinton agar plates with a spreader, and then wells of 8 mm diameter were aseptically punched using a sterile cork borer. The bottom of the wells was filled with 15 μL of preheated Mueller-Hinton agar to protect the extract against flowing under the agar. After the agar solidification, 80 μL of each extract solution of concentration of 2, 5, and 10 mg/mL was pipetted into the wells and cultured for 48 h at 37 °C, followed by calculation of an average values from five diameters of the inhibited growth zones of *P. larvae* strains under study from five replications. The well diameters were included as the inhibition zone. Additionally, susceptibility of the *P. larvae* strains to antibiotics was tested, using commercial discs of tetracycline (30 μg, Oxoid Ltd., Basingstoke, UK) and vancomycin (30 μg), according to the producer's procedure and recommendation of the National Committee for Clinical Laboratory Standards (NCCLS).

Secondly, the antimicrobial activity of the plant extracts and selected chemicals was also assessed in the tube dilution assay, allowing the determination of the minimal inhibitory concentration (MIC). The MIC values were accomplished in accordance with (i) the Clinical and Laboratory Standard Institutes (2011) protocols and (ii) recommendation of Andrews (2001). The stocks of extract solutions (2 mg/mL) were aseptically

diluted in double-concentrated Mueller-Hinton broth, receiving the arithmetic dilution series ranging from 0.5 to 1000 μg/mL in final volumes of 3 mL. Similarly, the stock solutions of tested chemicals were used for serial dilution from 0.0015 to 25 mM. Then, 30 μL of the bacterial culture of OD_{600}~0.2 was added to each tube with the extracts or chemical solutions. The bacteria were incubated for 48 h at 37 °C. The lowest concentration of the plant extracts which there was no visible growth was taken as the MIC. All the tests were repeated four times. The same procedure was also used to test the growth inhibition of tylosin (Sigma-Aldrich). In our experiment, the concentration of tylosin ranged from 0.001 to 500 μg/mL. This antibiotic is approved to use against *P. larvae* in the field (Wilson et al. 2015).

In both, the agar well diffusion test and the tube dilution assay (MIC determination), for the control *P. larvae* grown in Mueller-Hinton media (Mueller-Hinton agar in the agar well diffusion test or Mueller-Hinton broth in the MIC test) neither with the plant extracts nor with chemicals were chosen as positive controls. The media without bacteria incubated at the same conditions as the tested cultures were considered as negative controls. *P. larvae* strains growing onto/into media supplemented with 10% DSMO were used as a solvent control. Similar growth of the bacteria under study in Mueller-Hinton medium without DMSO and in Mueller-Hinton medium supplemented with 10% DMSO was observed in both tests, which allowed us to conclude that DMSO itself does not affect the *P. larvae* growth.

2.5. Statistical methods

The means and standard deviations (SD) were applied in descriptive statistics for continuous variables. To assess differences in antibacterial activity of plant extracts on *P. larvae* strains, two-way ANOVA analyses were performed, with diameter of zone of inhibition as an explained variable, and *P. larvae* strain and extract concentration as explanatory variables; p values were reported. Whereas to compare antibacterial activity between plant extracts on each of *P. larvae* strains, two-way ANOVA analyses were performed, with diameter of zone of inhibition as an explained variable, and plant extract and extract concentration as explanatory variables; p values were reported.

To assess effect of extract concentration on diameter of zone of inhibition, random-intercept mixed model was developed, with fixed effects for an extract-concentration interaction. Estimated coefficients with 95% confidence intervals and p values from the distribution were reported. The significance level was set at 0.05. Analysis was performed with R statistical software, version 3.4.0 (R Core Team (2017).

3. RESULTS

3.1. ERIC classification of the *P. larvae* isolates used in this study

The DNA pattern of the *P. larvae* isolates KB25, KB35, KB41, KB55, and the reference strain (LMG 09820) obtained by PCR with the ERIC primers showed identical ERIC patterns regardless of the origin of the samples from which they were isolated (Figure 1). Based on comparison between the obtained DNA fingerprints and those published previously (Genersch and Otten 2003; Genersch et al. 2006), the isolates were classified as the ERIC I genotype.

3.2. Chemical composition of extracts

In this work, the action on *P. larvae* of extracts from buds and young-leaved twigs of resin-giving plants (downy and silver birch, black poplar, and common aspen) of the boreal and temperate zones of Europe (Isidorov et al. 2016) was investigated. In different extracts of buds and young twigs, more than 390 organic compounds were registered by GC-MS with the relative content not less than 0.01% of TIC. For this reason, Table I gives only the group composition of extracts and the relative content of representatives of these groups.

The chemical composition of the diethyl ether extracts of ground buds (samples Bd-1−Bd-6) was rather close to the composition of previously investigated resins covering buds of corresponding

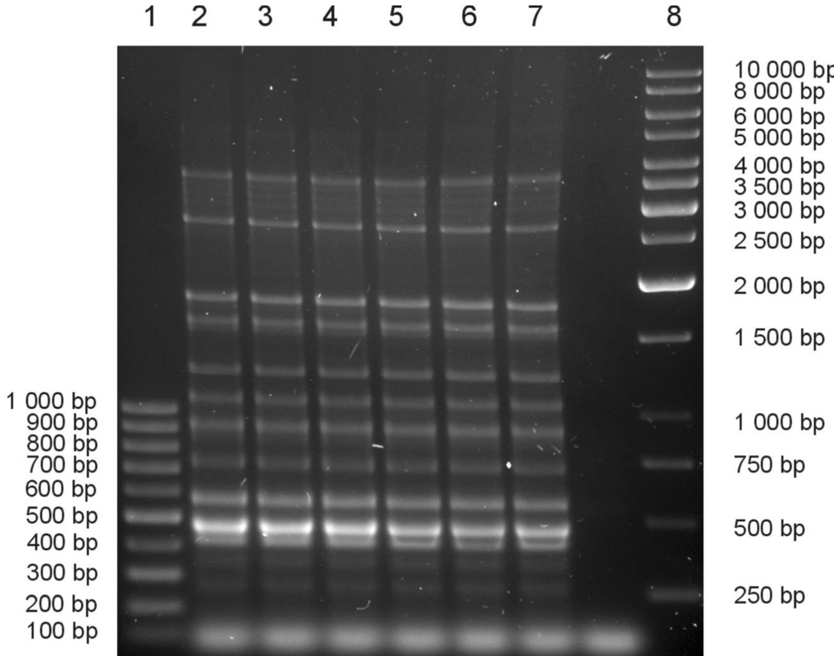

Figure 1 ERIC I patterns of *P. larvae* strains used in the study. Line: 1, GeneRuler DNA Ladders 100 bp (Thermo Fisher Scientific Com.); 2 and 7, LMG 09820; 3, KB25; 4, KB35; 5, KB41; 6, KB55; 8, GeneRuler DNA Ladders 1 kb (Thermo Fisher Scientific Com.).

trees: two species of birch, black poplar, and aspen (Isidorov and Vinogorova 2003; Isidorov et al. 2014a, b, 2016). However, some differences were observed at both qualitative and quantitative levels. For example, extracts Bd-3, Bd-5, and Bd-6 from ground silver birch and aspen buds contained a noticeable quantity (10–12% of TIC) of flavonoids (Table I) while their content in diethyl ether extract of resin covering silver birch and aspen bud surfaces was 0.9 and 4.2%, respectively (Isidorov et al. 2014b). Another difference concerns the content of catechin in extracts. Substantial quantities of this flavan-3-ol were detected in the extracts from ground buds of birches, poplar, and aspen (Table I); however, it was absent in the bud exudates collected by honey bees in the temperate zone of Europe (Isidorov et al. 2016).

Young twigs of two birch species were extracted successive with solvents of different polarity: non-polar *n*-hexane, poorly polar diethyl ether, and highly polar methanol. For this reason, the chemical composition of extracts was very different. The main components of the hexane extracts of both birch species were formed by relative poorly polar $C_{30}H_{48}O$, $C_{30}H_{50}O$, $C_{30}H_{52}O_2$, and $C_{30}H_{50}O_3$ triterpenoids. Although lupane series triterpenoids such as lupeol, betulinol, betulinic acid, and, related to latter, oleanolic acid are characteristic for both white birch species, representatives of dammarane series terpenoids (dammaradien-3-one and dipterocarpol) were only detected in extracts of silver birch. More contrasting is the chemical composition of the diethyl ether extracts of twigs. Downy birch extract contained 36.4% flavonoids, the content of which in silver birch extract was very low (0.3%). Different to the former species, the latter contained only trace amounts of sesquiterpenoids but large amounts of triterpenoids (60%).

Species-specific differences were also observed in the composition of glucosides, the most polar components extracted from birch twigs by methanol. In accordance with literature data (Laitinen et al. 2005), silver birch twigs contained a large amount of platyphylloside, (5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-glucopyranoside, which was completely absent in downy birch. In turn, specific to downy

Table I. Relative group composition (% of TIC) of extracts from plant material

Group of compounds	Buds' ether extracts						Downy birch twigs			Silver birch twigs		
	Downy birch Bd-1	Downy birch Bd-2	Silver birch Bd-3	Black poplar Bd-4	Aspen, spring Bd-5	Aspen, fall Bd-6	Hexane Ex-1	Ether Ex-2	Methanol Ex-3	Hexane Ex-4	Ether Ex-5	Methanol Ex-6
Flavonoids and chalcones	56.93	48.05	0.83	67.4	10.31	12.00	0.08	36.35	4.98	0.92	0.26	6.76
Pinocembrin	–	–	–	7.77	–	–	–	0.25	–	–	–	–
Pinocembrin chalcone	–	–	–	6.40	–	–	–	–	–	–	–	–
Pinostrobin chalcone	–	–	–	6.03	–	–	–	–	–	–	–	–
Chrysin	–	–	–	6.93	–	–	–	–	–	–	–	–
Galangin	–	–	–	10.3	–	–	–		–	–	–	–
Pinobanksin		–	–	3.97	–	–	–	–	–	–	–	–
Pinobanksin-3-acetate	–	–	–	14.23	–	–	–	–	–	–	–	–
5-Hydroxy-4′,7-dimethoxyflavone	2.17	2.71	–	–	–	–	–	4.68	–	–	–	–
Sakuranetin	12.47	14.61	0.13	0.08	3.12	4.17	0.08	14.05	0.95			–
Homoeriodictyol	6.45	4.69	–	–	Trace	1.98	–	1.96	–	–	–	–
Pectolinaringenin	7.94	4.60	–	–	–	–	–	2.54	–	–	–	–
Naringenin	1.14	0.76	–	0.08	2.40	2.13	–	–	–	–	–	–
Kaempferol	2.64	1.58	–	0.32	0.45	0.38	–	–	–	–	–	–
4-Methyl kaempferol	4.78	3.19	–	0.16	0.76	0.83	–	2.10	–	–	–	–
Apigenin	1.08	0.68	–	0.14	0.30	Trace	–	–	–	–	–	–
Quercetin	0.54	0.34	0.25	–	–	–	–	–	–	–	0.15	–
Catechin	0.29	0.25	0.70	0.12	1.85	1.28	–	–	2.76	0.27	1.70	4.66
Procyanidins B1 and B2	–	–	–	–	–	–	–	–	–	–	–	1.21
Cinnamic acids and esters	Trace	2.29	–	16.7	3.06	3.44	–	0.77	0.20	–	7.47	Trace
Hydroxycinnamic acids	Trace	0.48	–	1.22	3.01	2.00		–	0.20	–	–	Trace
Benzyl hydroxycinnamates	–	–	–	–		1.44		0.77	–	–	–	–
Pentenyl hydroxycinnamates	–	–	–	15.48	–	–		–	–	–	–	–

Table I (continued)

Group of compounds	Buds' ether extracts						Downy birch twigs			Silver birch twigs		
	Downy birch Bd-1	Downy birch Bd-2	Silver birch Bd-3	Black poplar Bd-4	Aspen, spring Bd-5	Aspen, fall Bd-6	Hexane Ex-1	Ether Ex-2	Methanol Ex-3	Hexane Ex-4	Ether Ex-5	Methanol Ex-6
Alkyl hydroxycinnamates	–	–	–	–	–	–		–	–	–	7.47	–
Phenylpropenoid sesquiterpenols	4.80	4.35	–	–	–	–	4.60	5.17	–	–	–	–
Phenylpropenoid glycerides	–	–	–	–	52.25	67.80	–	–	–	–	–	–
2-Acetyl-1,3-dicoumaroyl	–	–	–	–	19.32	21.30	–	–	–	–	–	–
2-Acetyl-3coumaroyl-1-caffeoyl	–	–	–	–	15.60	12.80	–	–	–	–	–	–
Other aromatics	0.57	–	0.37	0.58	0.35	0.91	8.56	0.01	2.38	–	2.18	0.55
Sesquiterpenoids	29.81	38.79	4.63	0.24	0.71	0.68	–	21.05	0.18	0.05	–	–
Triterpenoids	2.02	1.12	67.53	3.02	7.25	5.72	44.12	21.37	–	77.75	60.02	2.50
Dammaradien-3-one	–	–	5.00	–	–	–	–	–	–	8.38	6.11	–
Dipterocarpol	–	–	7.06	–	–	–	–	–	–	25.38	15.08	–
Lupeol	–	–	–	–	–	–	3.28	2.50	–	6.82	1.28	–
Betulinol	–	–	–	–	–	–	11.39	4.75		12.35	10.84	0.73
Betulinic acid	–	–	–	–	–	–	5.04	1.62		1.89	5.89	0.28
Oleanolic acid	–	–	–	–	–	–	9.31	1.09		2.43	8.61	0.57
Ursolic acid	–	–	–	–	–	–	0.30	–		0.35	0.63	–
Carbohydrates	–	–	–	–	–	–		–	6.99	–	–	6.78
Glucosides	Trace	Trace	Trace	0.05	0.25	0.66		–	83.60	–	3.98	71.98
Salicin	–	–	–	0.05	0.18	0.13	–	–		–	–	–
Salidroside	–	–	–	–	–	–	–	–	4.87	–	0.12	1.45
Rhododendrin (2 isomers)	–	–	–	–	–	–	–	–	16.73	–	3.86	14.64
Apiosylrhododendrin	–	–	–	–	–	–	–	–	1.00	–	–	–
Platyphylloside	–	–	–	–	–	–	–	–	–		–	43.80
Catechin 7-O-glucoside	–	–	–	–	–	–	–	–	52.10	–	–	2.10
Aliphatic acids and esters	1.13	0.73	11.28	0.11	15.28	12.0	19.94	5.62	–	10.06	14.03	0.58

Table I (continued)

Group of compounds	Buds' ether extracts						Downy birch twigs			Silver birch twigs		
	Downy birch Bd-1	Downy birch Bd-2	Silver birch Bd-3	Black poplar Bd-4	Aspen, spring Bd-5	Aspen, fall Bd-6	Hexane Ex-1	Ether Ex-2	Methanol Ex-3	Hexane Ex-4	Ether Ex-5	Methanol Ex-6
Aliphatic alcohols	0.53	0.38	0.58	0.47	0.58	0.47	4.77	1.71	0.80	2.66	7.44	0.11
Alkanes and alkenes	1.46	3.08	10.21	1.06	5.14	1.54	4.98	3.37	–	–	0.74	–

birch glucoside was apiosylrhododendrin (Santamour and Lundgren 1997). However, the main glucoside in the methanol extract of downy birch was catechine-7-O-D-glucopyranoside. To the best of our knowledge, it has not previously been identified in white birch tissue. Other highly polar components of methanol extracts from birch twigs were represented by catechin and its dimers, procyanidins B1 and B2.

3.3. Antibacterial activity

For testing the antibacterial activity of plant extracts, we used an agar well diffusion test and a tube dilution assay. As given in Table II, the highest anti-*P. larvae* activity was noted for bud extracts from *B. pendula* (Bd-3) and *P. nigra* (Bd-4). For these extracts at the concentration of 10 mg/mL, the inhibition zones varied from 27.2 ± 0.7 mm (KB25) to 30.5 ± 0.5 mm (KB55 and LMG 09820), and from 25.7 ± 0.9 mm (LMG 09820) to 30.2 ± 0.4 mm (KB25 for the *B. pendula* (Bd-3) and *P. nigra* (Bd-4) extracts), respectively. Among birch twig extracts, the highest activity was observed for hexane extracts from *B. pubescens* (the inhibition zones from 23.0 ± 0.6 to 30.4 ± 0.5 mm for KB55 and KB41, respectively) and hexane extracts from the *B. pendula* twigs (the inhibition zones from 28.0 ± 0.0 to 30.8 ± 1.2 mm for KB55 and LMG 09820, respectively). As presented in Table II, we found statistically significant differences in antibacterial effect of Bd-1, Bd-3, Bd-5, Ex-2, Ex-3, and Ex-5 between *P. larvae* strains (two-way ANOVA p value for *P. larvae* strains < 0.001 in each test), while antibacterial effect of Bd-4, Ex-1, and Ex-4 did not differ significantly between *P. larvae* strains. We also found statistically significant differences in antibacterial activity between extracts on each of *P. larvae* strain (two-way ANOVA p value for extract < 0.001 in each test). All *P. larvae* isolates under study and the reference strain LMG 09820 were susceptible to vancomycin and tetracycline, with the inhibition zones ranging from 25.7 ± 0.2 to 26.1 ± 0.1 mm for vancomycin and being > 40 mm for tetracycline (Table II).

Effect of extract concentration on diameter of zone of inhibition estimated in mixed model was

Table II. Average zones of inhibition (in mm ± standard deviation, $n = 5$) of selected plant extracts and commercial antibiotics against *P. larvae*

Sample		Extract concentration (mg/mL)	Average diameter of inhibition zone					Two-way ANOVA** p value
			KB25	KB35	KB41	KB55	LMG 09820	
Bud ether extracts								
Bd-1	*B. pubescens* buds	2	13.0 ± 0.0	13.4 ± 0.5	14.4 ± 0.5	13.2 ± 0.5	12.4 ± 0.5	< 0.001
		5	17.0 ± 0.0	17.2 ± 0.4	16.2 ± 0.4	18 ± 0.2	17.8 ± 0.7	
		10	22.2 ± 0.0	23.0 ± 1.1	26.0 ± 0.0	27.8 ± 0.7	21.8 ± 0.4	
Bd-3	*B. pendula* buds	2	16.0 ± 0.6	19.4 ± 0.5	18.4 ± 0.5	19.2 ± 0.7	21.0 ± 0.0	< 0.001
		5	21.0 ± 0.0	22.4 ± 0.5	25.0 ± 1.2	24.2 ± 0.9	22.6 ± 1.2	
		10	27.2 ±0.7	28.2 ± 0.4	30.0 ± 0.6	30.5 ± 0.5	30.5 ± 0.5	
Bd-4	*P. nigra* buds	2	19.4 ± 1.0	22.0 ± 0.4	23.2 ± 1.0	23.6 ± 0.8	23.4 ± 0.5	N.S. ($p = 0.146$)
		5	28.7 ± 0.5	25.4 ± 0.5	24.4 ± 0.5	22.4 ± 0.8	24.0 ± 0.6	
		10	30.2 ± 0.4	28.6 ± 0.8	28.4 ± 0.5	28.2 ± 0.4	25.7 ± 0.9	
Bd-5	*P. tremula* buds	2	10.8 ± 0.4	11.0 ± 0.0	11.2 ± 0.7	11.6 ± 0.5	9.0 ± 0.0	< 0.001
		5	14.4 ± 0.5	14.2 ± 0.4	15.8 ± 0.7	17.6 ± 0.8	14.0 ± 0.6	
		10	17.8 ± 0.7	18.4 ± 0.7	17.4 ± 0.5	19.0 ± 0.6	16.8 ± 0.7	
Birch young twigs extracts								
Ex-1	*B. pubescens*, hexane extract	2	17.0 ± 0.2	17.5 ± 0.4	16.2 ± 0.2	18.0 ± 0.2	17.8 ± 0.5	N.S. ($p = 0.304$)
		5	24.4 ± 0.5	24.2 ± 0.4	25.8 ± 0.7	27.6 ± 0.8	24.0 ± 0.6	
		10	28.8 ± 0.7	28.4 ± 0.7	30.4 ± 0.5	23.0 ± 0.6	27.8 ± 0.7	
Ex-2	*B. pubescens*, ether extract	2	12.3 ± 0.2	12.8 ± 0.4	11.8 ± 0.6	11.5 ± 0.2	12.0 ± 0.2	< 0.001
		5	18.2 ± 0.4	17.5 ± 0.6	16.9 ± 0.4	17.0 ± 0.5	18.0 ± 0.0	
		10	21.2 ± 0.2	21.6 ± 0.0	20.4 ± 0.4	20.7 ± 0.4	21.7 ± 0.5	
Ex-3	*B. pubescens*, methanol extract	2	n.a.	n.a.	n.a.	n.a.	n.a.	<0.001
		5	8.0 ± 0.0	8.6 ± 0.5	9.8 ± 0.4	9.0 ± 0.0	10.0 ± 0.0	
		10	11.2 ± 0.4	10.0 ± 0.0	10.2 ± 0.4	10.0 ± 0.0	11.0 ± 0.0	
Ex-4	*B. pendula*, hexane extract	2	15.8 ± 0.7	19.2 ± 0.4	19.0 ± 0.0	18.0 ± 0.6	18.2 ± 1.3	N.S. ($p = 0.589$)
		5	25.6 ± 0.8	24.6 ± 0.5	24.8 ± 0.9	25.8 ± 0.4	24.4 ± 0.5	
		10	29.0 ± 1.1	30.0 ± 0.0	29.4 ± 0.8	28.0 ± 0.0	30.8 ± 1.2	
Ex-5		2	12.8 ± 0.7	12.2 ± 0.4	14.0 ± 0.0	11.0 ± 0.6	12.2 ± 1.3	< 0.001

Table II (continued)

Sample		Extract concentration (mg/mL)	Average diameter of inhibition zone					Two-way ANOVA** *p* value
			KB25	KB35	KB41	KB55	LMG 09820	
	B. pendula, ether extract	5	15.6 ± 0.8	14.6 ± 0.5	16.8 ± 0.9	13.8 ± 0.4	15.8 ± 0.5	
		10	19.0 ± 1.1	20.0 ± 0.0	21.4 ± 0.6	18.0 ± 0.0	20.8 ± 0.9	
Ex-6	*B. pendula*, methanol extract	2	8.1 ± 0.5	n.a.	8.0 ± 0.0	n.a.	8.2 ± 0.5	N.A.
		5	9.0 ± 0.0	10.6 ± 0.5	9.8 ± 0.4	9.0 ± 0.0	10.0 ± 0.0	
		10	13.2 ± 0.4	14.0 ± 0.5	13.2 ± 0.4	15.0 ± 0	15.0 ± 0.0	
Two-way ANOVA[&] *p* value for the extracts			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	
Antibiotic								
Tetracycline (30 µg)			> 40	> 40	> 40	> 40	> 40	N.A.
Vancomycin (30 µg)			26.0 ± 0.1	26.0 ± 0.1	25.8 ± 0.2	26.0 ± 0.1	25.7 ± 0.2	N.A.

n.a. did not exhibit any antibacterial activity, *N.S.* non-significant, *N.A.* not applicable

**Two-way ANOVA for extract concentration and *P. larvae* strain, *p* values for *P. larvae* strain

[&] Two-way ANOVA for extract concentration and plant extract, *p* values for plant extract

Table III. Effect of selected plant extract concentration on diameter of zone of inhibition (mm) in mixed model fixed effects estimates

Variable*	Coefficient	95% CI	*p* value
(Intercept)	13.964	13.417–14.510	< 0.001
Bd-1	0.879	0.767–0.991	< 0.001
Bd-3	1.564	1.453–1.676	< 0.001
Bd-4	1.666	1.554–1.778	< 0.001
Bd-5	0.098	− 0.014–0.209	0.088
Ex-1	1.540	1.428–1.652	< 0.001
Ex-2	0.815	0.703–0.927	< 0.001
Ex-3	−0.464	− 0.575 to − 0.354	< 0.001
Ex-4	1.489	1.377–1.601	< 0.001
Ex-5	0.629	0.517–0.741	< 0.001

*Plant extract concentrations (in mg/mL) were used as a variable

significantly higher than zero for extracts Bd-1, Bd-3, Bd-4, Ex-1, Ex-1, Ex-4, and Ex-5. The greatest effect was observed for Bd-3, Bd-4, Ex-1, and Ex-4, for which the concentration increase of 1 mg/mL results in the increase in inhibition zone by about 1.5 mm. The results on the effect of extract concentration on diameter of zone of inhibition estimated in mixed model are given in Table III.

In the tube dilution assay, we determined the sensitivity of the wild isolates as well as the reference *P. larvae* strain LMG 09820 shown as the

Table IV. Minimal inhibitory concentration (MIC) of different extracts and tylosin for *Paenibacillus larvae* strains used in the study

Sample		MIC (μg/mL) for *P. larvae*				
		KB25	KB35	KB41	KB55	LMG 09802
Bud ether extracts						
Bd-1	*B. pubescens* buds (IV.2015)	7.8	7.8	7.8	7.8	15.6
Bd-2	*B. pubescens* buds (IX.2015)	7.8	7.8	7.8	7.8	15.6
Bd-3	*B. pendula* buds (IX.2015)	1.95	< 1.0	< 1.0	3.9	< 1.0
Bd-4	*P. nigra* buds (IX.2015)	7.8	7.8	7.8	15.6	7.81
Bd-5	*P. tremula* buds (IV.2015)	31.8	15.6	15.6	15.6	15.6
Bd-6	*P. tremula* buds (IX.2015)	31.8	15.6	15.6	15.6	15.6
Birch twigs extracts						
Ex-1	*B. pubescens*, hexane extract	3.9	3.9	< 1.0	< 1.0	7.8
Ex-2	*B. pubescens*, ether extract	15.6	15.6	31.8	31.8	7.8
Ex-2	*B. pubescens*, methanol extract	62.5	62.5	125.0	125.0	62.5
Ex-3	*B. pendula* young twigs, hexane extract	< 1.0	< 1.0	< 1.0	3.9	< 1.0
Ex-4	*B. pendula* young twigs, methanol extract	31.8	31.8	31.8	15.6	7.8
Ex-5	*B. pendula*, ether extract	31.8	31.8	31.8	15.6	7.8
Ex-6	*B. pendula*, methanol extract	31.8	31.8	62.5	62.5	62.5
Tylosin		< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

Table V. Minimal inhibitory concentration of selected plant derived compounds for *Paenibacillus larvae*

			MIC (µg/mL) for *P. larvae* strains				
Triterpenoid	Formula	MW	KB25	KB35	KB41	KB55	LMG o9820
Dipterocarpol		442.7	34.5	34.5	34.5	17.3	17.3
Lupeol		426.7	33.3	8.5	8.55	16.6	4.3
Betulin		442.7	69.1	17.3	17.3	17.3	<4.4
Betulinic acid		456.7	9.1	18.3	9.1	4.6	9.11
Ursolic acid		456.7	17.8	17.8	17.81	9.1	<4.6
Oleanolic acid		456.7	35.6	17.8	17.81	35.6	<4.6
Quercetin		302.3	295.0	-*	-	-	1180.9
(+)-Catechin		290.3	1814.02	-	-	-	3625.4
Salicin		272.2	>10000	-	-	-	>10000
Arbutin		268.3	7157.5	-	-	-	>10000
Rhaponticin		420.4	6560.0	-	-	-	6560.0
Baicalin		446.4	871.0	-	-	-	223.2

*- not tested

*- not tested

MIC value for each extract used in the study. The results are presented in Table IV. In spite of the great differences in the chemical composition, all tested extracts significantly inhibited the growth of *P. larvae*, with MIC from < 1 to 125 μg/mL. The most active was the ether extract from silver birch buds, as well as the *n*-hexane extracts of silver and downy birch twigs, which showed MIC values from below 1.0 to 3.9 μg/mL. Significantly lower anti-*P. larvae* activity was demonstrated in methanol extracts, with MIC values from 31.8 to 125 μg/mL. In the agar well diffusion test, the antimicrobial activity of the extracts was confirmed. The MIC value for tylosin was assessed to be lower than 1 μg/mL (Table IV).

To examine the causes of observed differences in anti-*P. larvae* activity, the antimicrobial properties of selected pure chemicals (triterpenoids, flavonoids, and glycosides) were determined (Table V). The most potent anti-*P. larvae* compounds were triterpenoids while the flavonoids and glucosides tested demonstrated considerably weaker activity (quercetin, catechin, baicalin) or were entirely inactive (salicin, arbutin, rhaponticin).

4. DISCUSSION

The isolates KB25, KB35, KB41, and KB55 used in this study were previously classified as *P. larvae* (Isidorov et al. 2017) based on the colony shape (round, slightly whitish), lack of catalase, formation of spiral forms in the Plagemann test, and the 16S RNA sequences (Neuendorf et al. 2004; Plagemann 1985). In this study, we determined that all of them belong to the ERIC I genotype. According to Genersch (2010), ERIC I and ERIC II are the two most important genotypes of *P. larvae* which can be isolated from AFB-diseased colonies in Europe. These two genotypes differ in their ability to cause disease at the larval stage. ERIC I and ERIC II needed 12 and 7 days, respectively, to kill all infected larvae. Hence, larvae infected by the fast-killing genotype ERIC II die for the most part before cell capping and can be removed (prior to production of infectious spores) by nurse bees as part of the social immune defense (Spivak and Reuter 2001; Genersch 2010). Removal of capped dead prepupae killed by ERIC I, as well as those containing huge amount of spores in a ropy mass from cell bottom, is less efficient and can favor disease transmission within a colony. For this reason, the majority of AFB cases registered in Poland are probably caused by the slower killing *P. larvae* ERIC I genotype.

The results of microbiological assays presented in Tables II, III, and IV allow us to draw some preliminary conclusions. The first consists of stating the high efficiency of extracts from buds of resin-giving plants against *P. larvae*. In principle, the high antimicrobial activity of these extracts may not be surprising because their chemical composition is very close to the composition of honey bee propolis. The antimicrobial activity of honey bee products such as propolis and honey is attributed to flavonoids and phenylcarboxylic and hydrocinnamic acids: the higher the content of these phenolic compounds, the stronger the antibiotic properties of propolis or honey (Burdock 1998; Chen et al. 2018; Hegazi et al. 2000; Da Silva Filho et al. 2008; Kujumgiev et al. 1999; Popova et al. 2017; Wilson et al. 2017). In accordance with this conception is the observation of higher anti-*P. larvae* activity of extracts from *B. pubescens* and *P. nigra* buds compared to the activity of *P. tremula* bud extracts: the latter contains about five times less flavonoids (Table I). However, the highest activity against *P. larvae* was demonstrated by the extract from *B. pendula* buds with a relative small concentration (10.5% of TIC) of flavonoids. The principal components of this material (ca. 68% of TIC) are very complex and difficult to identify mixture of relatively poorly polar triterpenoids. Hence, the second conclusion is that the anti-*P. larvae* activity of the investigated plant extracts is connected in a certain way with the polarity of their constituents.

This hypothesis agrees with the MIC values presented in the second part of Table IV. It can be seen that the ether extract of downy birch twigs (Ex-2), with a relatively high content of flavonoids (36.5% of TIC), demonstrated lower anti-*P. larvae* activity than the hexane extract Ex-1 which contained only trace amounts of flavonoids. However, the tested *P. larvae* isolates were most susceptible to (nearly deprived of phenolics) the hexane extract from silver birch twigs (Ex-4). On the other hand, methanol extracts from downy

birch (Ex-3) and silver birch twigs (Ex-6) were substantially less active than both hexane extracts. Diethyl ether extracts Ex-2 and Ex-4 demonstrated intermediate activity.

The chemical composition of these three groups of extracts was highly contrasting. Highly polar methanol extracts from twigs contain highly polar hydrophilic compounds: carbohydrates and glucosides (Abyshev et al. 2007; Julkunen-Tiitto et al. 1996), while the poorly polar ether and non-polar *n*-hexane extract less polar and relatively lipophilic methoxylated flavonoids (sakuranetin, homoeriodictyol, pectolinaringenin, 4-methylkaempherol) and triterpenoids.

It is interesting to note that Boligon et al. (2013) evaluated the effect of crude extract and fractions of *Scutia buxifolia* against six *Paenibacillus* species, including *P. larvae*. It was found that the non-polar dichloromethane fraction showed the best MIC value (1.56 mg/mL), followed by moderately polar ethyl acetate (6.25 mg/mL) and polar *n*-butanol fractions (25.0 mg/mL). Kujumgiev et al. (1999) also noted that a decrease of the polar phenolic compound content in propolis was not accompanied by a decrease of activity against *Staphylococcus aureus.* The authors speculated that triterpenes, making up about 18% of the "balsam" of Egyptian propolis, might play a role in its antimicrobial properties. In a very recent study, Wilson et al. (2017) have isolated from American propolis 11 structurally related 3-acylpinobanksins (dihydroflavonols with non-substituted B ring) and characterized their anti-*P. larvae* activity. The antimicrobial action was related to acyl group length where less polar compounds (with longer acyl group) were better inhibitors of *P. larvae.* All these findings are consistent with our observation of anti-*P. larvae* action of investigated plant extracts.

In order to examine the relationship between the polarity of different compounds and their anti-*P. larvae* activity, we determined the MIC values of selected triterpenoids, flavonoids, and glucosides (Table V). These experimental data demonstrate for the first time the high activity against anti-*P. larvae* of triterpene alcohols and acids, but the lower activity of more polar flavonoids and, especially, highly polar glucosides. It is remarkable that baicalin containing a hydrophobic fragment in its molecule (non-substituted B ring) was much active than other glucosides. It can be assumed that high lipophilicity enables them to partition the lipids of the bacterial cell membrane and/or connect with the hydrophobic parts of proteins embedded in the cytoplasmic membrane, disturbing their structures (Burt 2004; Sikkema et al. 1995).

5. CONCLUSION

Antibiotics in the bee keeping are legally banned in many countries and this calls for an alternative control of AFB that do not contribute for the *P. larvae* resistance. Our study demonstrates that some plant extracts and plant-derived compounds (such as triterpenoids) have a high potential to control growth of these bacteria. The subsequent experiments will be devoted to oral toxicity of the more promising extracts to adult worker honey bees and field efficacy of administration.

AUTHOR CONTRIBUTIONS

VI—plant material collection, sample preparation, and GC-MS analysis; KB—*P. larvae* isolation and characterization; AS—statistical analysis; GZ and IS—microbiological investigationsFunding information

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Activité d'extraits de plantes sélectionnés contre l'agent pathogène de l'abeille mellifère *Paenibacillus larvae*

Abeille domestique / *Paenibacillus larvae* / extraits de plantes / activité antimicrobienne

Aktivität bestimmter Pflanzenextrakte gegen das Honigbienenpathogen *Paenibacillus larvae*

Honigbiene / *Paenibacillus larvae* / Pflanzenextrakte / antimikrobiale Aktivität

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