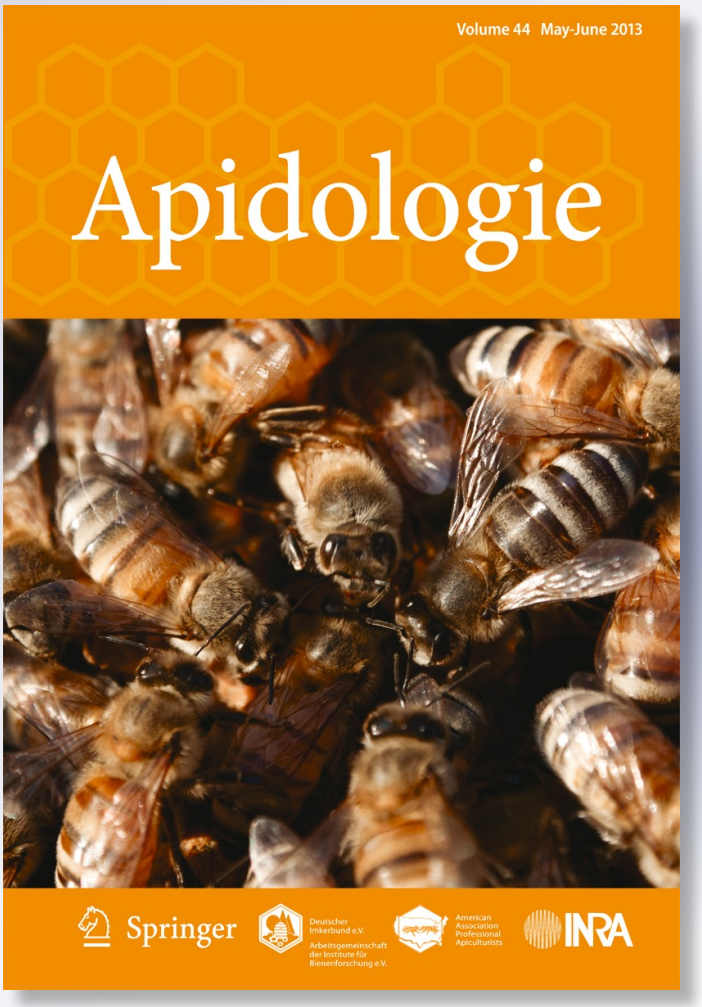
New anti-Paenibacillus larvae *substances purified from propolis*

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Original article

New anti-*Paenibacillus larvae* substances purified from propolis

Katarina Bilikova[1], Milena Popova[2], Boryana Trusheva[2], Vassya Bankova[2]

[1]Department of Molecular Apidology, Institute of Molecular Biology, Slovak Academy of Sciences, Dubravska cesta 21, SK-845 51 Bratislava, Slovakia
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 9, 1113 Sofia, Bulgaria

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Abstract – Propolis plays an important role in the exogenous defense of honeybee colony against pathogens. However, the studies dealing with the activity of propolis against bee pathogens are scarce. Poplar propolis extracts demonstrated promising activity against *Paenibacillus larvae*, the causative agent of American foulbrood. From the same propolis, five individual components and a mixture of caffeates were isolated, and their structures confirmed by spectroscopic data. Among the isolated propolis constituents are flavonoids, ferulic acid esters, and the oxylipin 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid, newly identified as propolis component. These substances were tested for their activity against *P. larvae* strains. The most active constituents were pinocembrin, 3-*O*-acetyl pinobanksin, and the caffeate mixture. This is the first communication of antimicrobial activity of individual propolis constituents against *P. larvae*; their important advantage is the fact that they are naturally present in the hive.

poplar propolis / anti-AFB agents / pinocembrin / 3-*O*-acetyl pinobanskin / 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid

1. INTRODUCTION

Propolis is one of the most fascinating materials produced by bees, both building material and defensive substance. Bees make use of the mechanical properties of this resinous product by applying it for blocking holes and cracks, repairing combs, strengthening the thin borders of the comb, etc. (Ghisalberti 1979). On the other hand, they make use also of its biological action: bee glue contains the putrefaction of "embalmed" intruders, killed in the hive, and it is responsible for the lower incidence of bacteria and molds within the hive than in the atmosphere outside (Ghisalberti 1979). The action against infections is an essential characteristic of propolis and this fact has been recognized and used by human beings since ancient times. The effectiveness of propolis and its individual constituents against different human pathogens, such as bacteria, fungi, and viruses, has been proved in numerous studies, published in the last four to five decades (reviews: Burdock 1998. Kujumgivev et al. 1999; Banskota et al. 2001; Bankova 2005; Sforcin and Bankova 2011; Shruthi and Suma 2012). Somewhat surprisingly, although propolis is well-known to be "the chemical weapon of bees against infections", the studies dealing with the action of propolis substances against bee pathogens are scarce. A recent study of Simone et al. (2009) has revealed the role of

Corresponding author: V. Bankova, Bankova@orgchm.bas.bg
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propolis in bees'social immunity. In hives sprayed with propolis solution, a lower expression of two honeybee immune-related genes was observed, compared to control colonies. In addition, the propolis treated colonies had lower bacterial loads than controls (Simone et al. 2009). In the last years, there is an emerging interest in the potential of natural products (Flesar et al. 2010) and especially of propolis to combat bee pathogens. The replacement of pesticides and antibiotics in beekeeping by propolis or its constituents is an appealing prospect (Antúnez et al. 2008; Bastos et al. 2008; Damiani et al. 2010; Simone-Fimdstrom and Spivak 2010; Mihai et al. 2012). Several research groups have performed in vitro tests for the activity of propolis total extracts against *P. larvae* using zone inhibition assays: disc diffusion method (Antúnez et al. 2008; Bastos et al. 2008) and agar diffusion techniques (Bastos et al. 2008; Mihai et al. 2012); or determining minimal inhibitory concentration (MIC) by observation of turbidity (Antúnez et al. 2008). In all those experiments propolis inhibited the growth of *P. larvae*.

Our preliminary antibacterial tests with propolis extracts for their activity against *P. larvae* gave promising results. For this reason, in the search of natural products useful in prophylactic and treatment of bee diseases, we decided to look for constituents of propolis extracts with significant activity against *P. larvae*. The aim of this work was to show antimicrobial effect of pure propolis compounds on microplate grow inhibition assay and reveal their potential as an alternative of classical antibiotics used in prevention of honeybee colony against American foulbrood disease (AFB).

2. MATERIALS AND METHODS

2.1. Isolation and identification of propolis constituents

General ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz), Bruker AV 600; spectra were taken in $CDCl_3$ (deuterated chloroform). MS was performed on a Hewlett Packard 5972 mass spectrometer, ionization voltage 70 eV. Column and vacuum liquid chromatography (VLC) were performed on Silica gel 60 (Merck, 63–200 μm), and Polyamide 6 (for column chromatography, Fluka). Analytical thin-layer chromatography (TLC) was performed on Silica gel 60 F_{254} plates (Merck). Preparative thin-layer chromatography was performed on Silica gel $60F_{254}$ glass plates (Merck, 20×20 cm, 0.25 mm). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with vanillin–sulfuric acid in methanol (5:95 (*w*/*v*) vanillin/methanol solution, freshly mixed with a 5:95 (*v*/*v*) sulfuric acid/methanol solution), followed by heating at 100 °C.

Propolis extraction Propolis, collected in 2009 in the region of the town of Elena, Bulgaria (500 g), was cut into small pieces and extracted with 70 % ethanol (1:10, *w*/*v*) for 24 h two times. Ten milliliters of the ethanol extract (PET) was evaporated to dryness and used in antibacterial tests. PET was concentrated in vacuo to reduce the volume by one half and then extracted successively with petrol ether (40–60 °C) (three times) and ethyl acetate (three times). The petrol ether extracts were combined and evaporated in vacuo to dryness to give 40 g dry residue (PEE), 1 g of it was used in antibacterial tests. The ethyl acetate extract was evaporated in vacuo to dryness to give 274 g dry residue (PEA), and 1 g was used in antibacterial tests.

Isolation and identification of compounds The dried petrol ether fraction was subjected to vacuum liquid chromatography on silica gel, mobile phase petrol ether–$CHCl_3$ (100 % petrol ether to 50 % petrol ether) and 11 fractions (A–K) were obtained. Fractions containing one major constituents (according to TLC) were subjected to further purification.

Fraction J was subjected to VLC on polyamide eluted with $CHCl_3$–MeOH (from 100 % $CHCl_3$ to 50 % $CHCl_3$) to yield 11 fractions. Fraction J2 was re-chromatographed on a silica gel column eluted with petrol ether—EtOAc (from 3 to 50 % EtOAc), and 12 fractions were collected.

Fractions F and G were combined and subjected to column chromatography on silica gel, mobile phase petrol ether—EtOAc (from 2 % EtOAc to 25 % EtOAc), 30 fractions were obtained [(F+G)1–(F+G)30]. Fractions (F+G)24 and (F+G)25 were combined and after final purification on a silica gel LoBar column (Size B) with mobile phase pure $CHCl_3$ yielded 125 mg benzyl ferulate **B1**, identified by comparison of Rf values, MS, and NMR spectra with an authentic sample.

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Fraction (F+G)21 was re-chromatographed on a silica gel LoBar column (Size B) eluted with $CHCl_3$ - EtOAc (from 1.5 % EtOAc to 50 % EtOAc) and fraction (F+G)21-3 gave 239 mg pentenyl ferulate **B2** (3-methyl-2-butenyl ferulate : 3-methyl-3-butenyl ferulate 2:0.5, ratio according to ^{1}H-NMR). Identity was confirmed by comparison of MS and NMR spectra with authentic samples.

Fraction J2-9 contained 230 mg of 9-oxo-10(*E*)-12 (*Z*)-octadecadienoic acid **B4**. The identity of the compound was confirmed by comparison of its spectral data (MS, ^{1}H-, and ^{13}C-NMR) with literature data (Kuklev et al. 1997).

Fractions J2-4 and J2-5 were combined and re-chromatographed on a silica gel column eluted with petrol ether – EtOAc (from 2 to 50 % EtOAc), and fraction 5, after recrystallization from acetone, afforded 67 mg pinocembrin **B5**, identified by comparison with an authentic sample (Rf value, NMR spectra).

A part of the dry ethyl acetate extract (30 g) was subjected to VLC on Polyamide eluted with $CHCl_3$–MeOH–EtCOMe (20:2:1; 20:4:2; 20:5:3, 20:8:4, 20:10:5, 20:12:6–1,200 mL each) and 21 fractions were obtained (EA–EU). Fractions EC and ED were combined and re-chromatographed on a Polyamide column, mobile phase $CHCl_3$–MeOH (from 0.5 % MeOH to 50 % MeOH) and 16 fractions were obtained. Fractions (EC+ED)8–(EC+ED)11 were combined and further purified on a silica gel Lobar column (size B) eluted with $CHCl_3$–EtOAc (2 EtOAc to 30 % EtOAc) to yield 87 mg of a mixture of caffeic acid esters **B3** (isopentenyl, dimethylallyl, phenetyl, and benzyl caffeates 1:1.9:1.3:0.9, according to ^{1}H-NMR data). Identity was confirmed by comparison of MS and NMR spectra with authentic samples.

Fractions (EC+ED)5 was purified on silica gel Lobar column (size B) eluted with petrol ether—EtOAc (1 % EtOAc to 15 % EtOAc) and 53 mg pinobanksin 3-*O*-acetate **B6** were obtained, its identity was confirmed by comparison with an authentic sample (Rf value, NMR spectra).

2.2. Bacterial strains and growth conditions

Bacterial strains were obtained from Swedish collection of microorganism (provided by Dr. Eva Forsgren, Swedish University of Agricultural Sciences, Uppsala, Sweden) under the designations *P. larvae* subsp. *larvae* (ERIC I) swe159/97 and *P. larvae* subsp. *pulvifaciens* (ERIC II) swe 26/02 (classification of *P. larvae* strains according to Genersch et al. 2006). The strains were stored at −70 °C on MYPGP broth with 20 % (*v*/*v*) glycerol stocks before use. The cultivation/assay medium MYPGP broth or agar contained (per liter) 10 g Müller–Hintonon broth, 15 g yeast extract, 3 g K_2HPO_4, 2 g glucose and 1 g sodium pyruvate (Merck, Germany) supplemented with 0.1 μg/mL thiamine hydrochloride (Sigma, USA). Bacteria were grown at 36 °C for 48 h. Optical density was measured at 595 nm.

2.3. Inhibition zone assay

The bacterial strains in exponential phase of the growth were used for inoculation of MYPGP with 0.5 % agar at density of 10^5CFU/mL for preparing plates for inhibition zone assay. Then, 5 μL of crude propolis extracts PET, PPE, and PAE at the concentration of 20, 10, 5, and 2 mg/mL and pure propolis compounds B1, B2, B3, B4, B5, B6 at 5 mg/mL in DMSO each were applied into 2 mm diameter holes in the prepared plates and incubated at 36 °C for 48 h. Tetracycline hydrochloride in concentrations of 0.5, 1, and 2 μg/mL was used as a positive control and DMSO as a negative control of antimicrobial effect. Each plate of the assay was repeated twice.

2.4. Determination of minimal inhibitory concentration

The MIC of propolis compounds were determined using broth microdilution method. Stock solution of propolis compounds was prepared, as follows: a solution in DMSO at concentration of 60 mg/mL was first diluted with 50 mM NaOH to 20 mg/mL and then cultivating medium was added to concentration of 2 mg/mL. This stock solution was used for serial dilution in a 96-wells microtiter microplate from 1 to 500 μg/mL, 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/mL. For the broth microdilution test, 5 μL of bacterial suspension in exponential phase of the growth was added to the wells of a sterile 96-well microtiter plate already containing 145 μL of twofold serially diluted propolis compounds in growth medium. Control wells were prepared with culture medium and bacterial suspension only. Three wells

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of the microtitre plate were used for each concentration of tested propolis compounds as well as for control sample. Incubation of the microplate was done for 48 h in the cultivation conditions described above. Absorbance (A) was measured at 595 nm by BioTek Microplate Spectrophotometer, PowerWave^{TM} XS. Data were evaluated by Gen5 Microplate Software (BioTek Instruments, Inc. USA). For determination of MIC, the average value of absorbance at 595 nm was calculated.

3. RESULTS

Among the isolated propolis constituents are two ferulic acid esters (benzyl ferulate **B1** and pentenyl ferulate **B2**), two flavonoid aglycones (pinocembrin **B5**, 3-*O*-acetyl pinobanskin **B6**), and one oxygenated fatty acid, 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid **B4**, newly identified as propolis component. We isolated also a compound mixture **B3**, hard to be preparatively separated, consisting of caffeic acid esters: isopentenyl caffeate, dimethylallyl caffeate, phenetyl caffeate (CAPE), and benzyl caffeate The exact ratio of the constituents was 1:1.9:1.3:0.9, according to ^{1}H-NMR data.

For first test of antimicrobial activity of propolis extracts we used Inhibition zone assay. The sensitivity of *P. larvae* strains swe 159/97 (ERIC I) and swe 26/02 (ERIC II) to propolis extracts (PET, PPE, PAE) and isolated propolis constituents (Figures 1 and 2) in DMSO were tested. As it is shown in Figure 2, the most effective in this assay were **B5** and **B1**. Antimicrobial effect of **B5** and **B6** against swe 26/02 was comparable after 24 h of incubation (Figure 2, panel c), after 48 h the **B5** kept the antimicrobial activity while in case of **B6** the activity fall down significantly (Figure 2, panel c).

Propolis extracts are usually well soluble in organic solvents but only weakly in water, and it is difficult to find conditions for their solubility in aqueous solvents which is required for growth inhibition assay in liquid medium. In the case of some propolis compounds, it is possible to achieve higher solubility in aqueous solutions. Under the conditions of our experiment (see Section 2), the tested propolis compounds were solubilized and used for growth inhibition of *P. larvae* strains in liquid medium. Obtained results of MICs determined after 12, 24, 36, and 48 h of incubation are presented in Table I.

The most active compounds were the flavonoids pinocembrin **B5** and 3-*O*-acetyl pinobanksin **B6**, and the caffeate mixture **B3**. Time dependant growth inhibition curves for *P.*

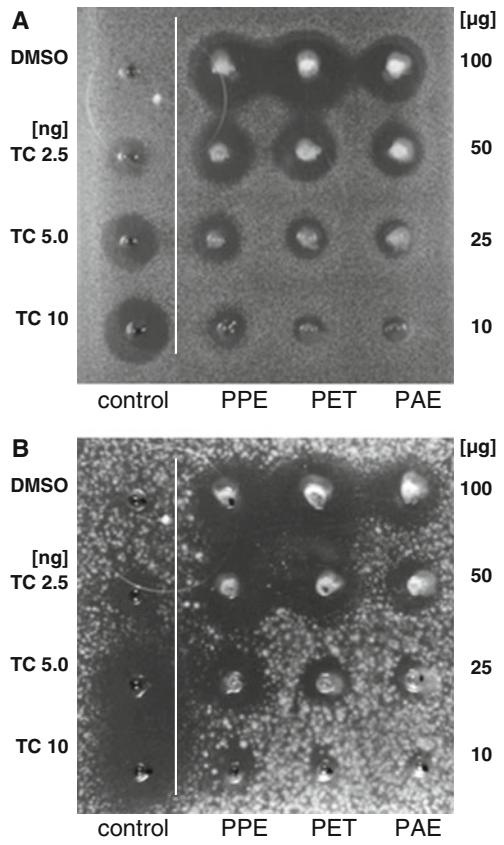
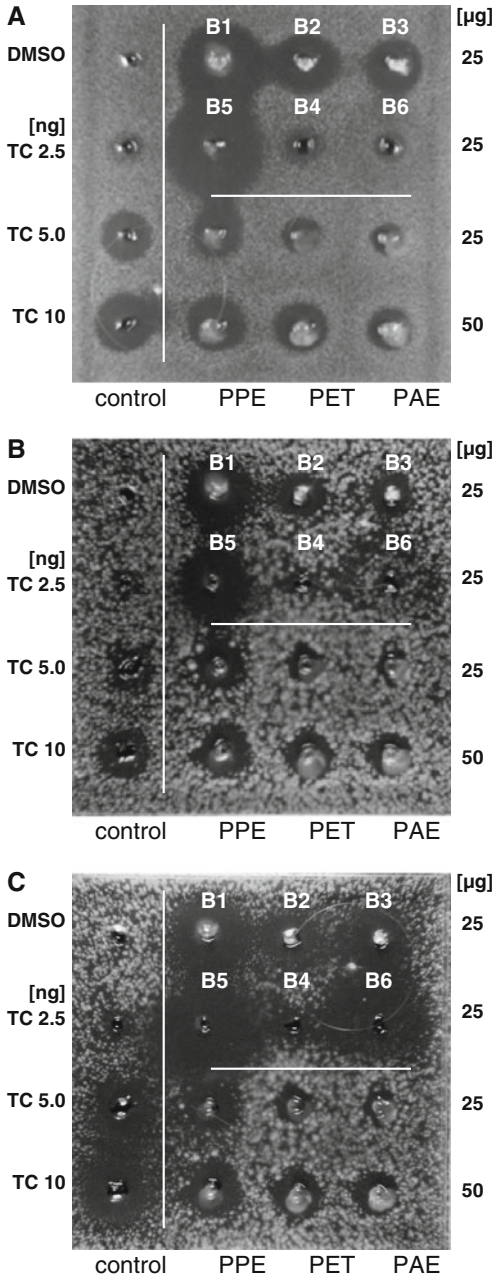


Figure 1. Antimicrobial activity of propolis extracts to *P. larvae* using inhibition zone assay on MYPGP agar plates after 48 h of incubation at 36 °C. Panel **a** *P. larvae* swe 159/97 (ERIC I). Panel **b** *P. larvae* swe 26/02 (ERICII). Samples in DMSO (total amount applied in 5 µL): *PET* total extract of propolis; *PPE* petrol ether fraction of total extract of propolis; *PAE* ethylacetate fraction of total extract; *TC* tetracycline hydrochloride, as positive control; *DMSO* dimethylsulfoxide, as negative control of grow inhibition.

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◀**Figure 2.** Antimicrobial activity of pure propolis compounds to *P. larvae* using inhibition zone assay on MYPGP agar. Panel **a** *P. larvae* swe 159/97 (ERIC I) and panel **b** *P. larvae* swe 26/02 plates, after 48 h of incubation at 36 °C. Panel **c** *P. larvae* swe 26/02 (ERIC II) after 24 h of incubation at 36 °C. Samples in DMSO (total amount applied in 5 µL): **B1**, benzyl ferulate; **B2**, pentenyl ferulate; **B3**, caffeate mixture average; **B4**, 9-oxo-10(*E*),12(*Z*)-octadecanoic acid; **B5**, pinocembrin; **B6**, pinobanksin-3-*O*-acetate; *PET* total extract of propolis; *PPE* petrol ether fraction of total extract of propolis; *PAE* ethylacetate fraction of total extract; *TC* tetracycline hydrochloride, as positive control; *DMSO* dimethylsulfoxide, as negative control of grow inhibition.

larvae swe 159/97 (ERIC I) and *P. larvae* swe 26/02 (ERIC II) in presence of pinocembrin **B5** at different concentrations are represented in Figure 3. A clear dose-dependent antimicrobial effect can be observed.

4. DISCUSSION

Pinocembrin **B5**, 3-*O*-acetyl pinobanskin **B6**, the caffeic acid esters (isopentenyl, dimethylallyl, penethyl and benzyl caffeate), and the ferulic acid esters (benzyl and pentenyl ferulate **B1** and **B2**) are well-known constituents of poplar type propolis. Their activity against human bacterial pathogens has been confirmed in many studies (Metzner et al. 1979; Kujumgiev et al. 1993; Banskota et al. 2001; Darwish et al. 2010). Our data demonstrate for the first time their activity against the bee pathogen *P. larvae*. It is interesting to note that in a very recent study, Mihai et al. (2012) have established the importance of flavanones and dihydroflavonols for the activity of propolis total extract against *P. larvae*. Our results show the significant activity of individual propolis constituents of this class (pinocembrin and 3-*O*-acetyl pinobanskin) and thus confirm their findings.

The high inhibition observed in this study for the caffeate mixture is not surprising. One of its constituents, the CAPE, is the most studied propolis constituent; it possesses well-documented and valuable biological activities, such as antitumor, antibacterial, antiviral, antioxidant (Bankova 2009).

On the other hand, the oxygenated fatty acid **B4** 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid, is of special interest because it is identified in propolis for the first time. This compound belongs to the class of oxylipins, widespread

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Table I. Minimal inhibitory concentration (MIC) of pure propolis compounds for *P. larvae* (ERIC I) and *for P. larvae* (ERIC II) determined after 12, 24, 36, and 48 h of incubation in MYPGP medium.

Sample no.	Compound	MW	MIC [μg/mL]							
			P. larvae subsp. *larvae* swe 159/97 (ERIC I)				*P. larvae* subsp. *pulvifaciens* swe 26/02 (ERIC II)			
			12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h
B1	Benzyl ferulate	284	250	250	500	500	250	500	500	1,000
B2	Pentenyl ferulate	276	125	250	500	500	250	500	500	1,000
B3	Caffeate mixture (average)	270	31.25	62.5	125	125	31.25	62.5	125	125
B4	9-oxo-10(*E*),12(*Z*)-octadecdienanoic acid	294	250	500	500	1,000	250	500	500	1,000
B5	Pinocembrin	256	31.25	62.5	62.5	62.5	62.5	125	125	250
B6	Pinobanksin-3-*O*-acetate	314	31.25	31.25	62.5	125	62.5	125	125	250

in aerobic organisms including plants, animals, and fungi (Iriti and Faoro 2007). The C_{18} oxylipins are typical for the plant kingdom (Mosblech et al. 2009) and so the most probable source of the identified 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid **B4** is the propolis source, the black poplar. Noteworthy, plant oxylipins are involved in resistance to various microbial pathogens and other pests. The 9-oxo-10(*E*)-12(*Z*)-octadecadienoic acid **B4** itself has recently been found to possess significant activity against bacteria and fungi (Prost et al. 2005).

The finding that all tested propolis constituents inhibit the growth of *P. larvae* to different extents is in agreement with the conclusions of Mihai et al. (2012) that interaction effects among the various chemical compounds in propolis is of the essence for its antibacterial effects against honeybee pathogens.

As it is obvious from the data in Table I, the strain *P. larvae* swe 159/97 (ERIC I) is the most susceptible one to propolis constituents.

Recently, it has been found that propolis collection in the colony increases after challenging with a fungal pathogen, which is an unambiguous proof for the defensive role of propolis (Simone-Finstrom and Spivak 2012). The antibacterial compounds identified in our study are regular components of propolis presented in honeybee colony and this is an important advantage in comparison to the banned antibiotics. However, their protective properties are not readily available to honeybees in their natural food (honey, royal jelly) because of low solubility in water. In the present work, we showed antimicrobial effect of some propolis compounds against two strains of *P. larvae*, the causal pathogen of AFB, in liquid medium. Minimal inhibitory concentrations determined in different times of incubation can provide us information about time period for usage of these compounds for feeding honeybee colony by saccharose syrup as substituent of classical antibiotics like tetracyclines, thylosine, etc., already prohibited in beekeeping. This way of application of propolis constituents seems promising, taking into consideration the

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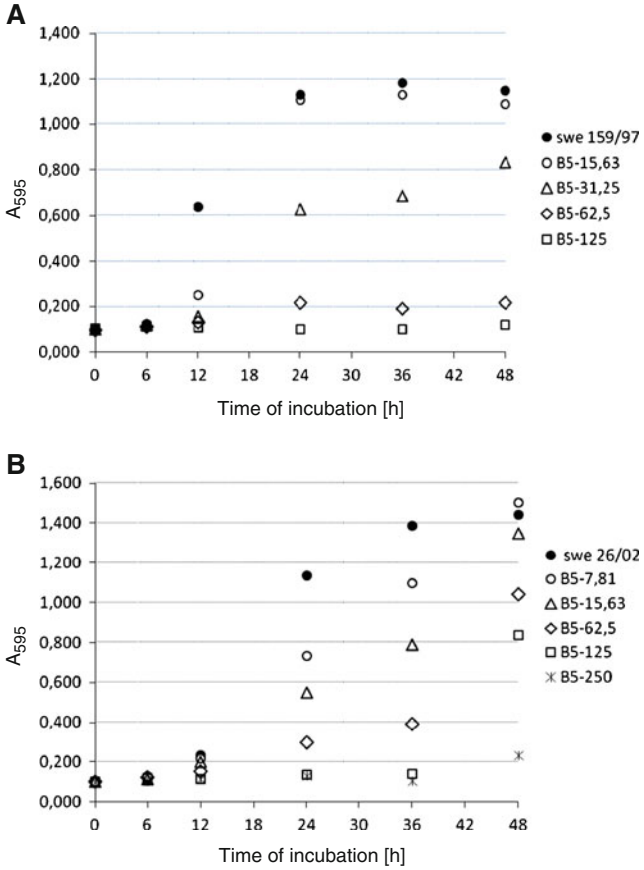


Figure 3. Growth inhibition of *P. larvae* swe 159/97 (ERIC I) (Panel **a**) and *P. larvae* swe26/02 (ERIC II) (Panel **b**), in liquid MYPGP medium in presence of pinocembrin (**B5**). Concentration in micrograms per millilters. Growth of the *P. larvae* strains without addition of antimicrobial agent was used as control.

results of Antúnez et al. (2008): they demonstrated the lack of oral toxicity of propolis extract to bees even at concentration 50 % in the sugar syrup. Nevertheless, toxicity of individual compounds should be tested, because it is not necessarily the same as the one of total propolis extracts.

Demonstration of antimicrobial activity of individual propolis compounds by growth inhibition of honeybee pathogenic bacteria in liquid medium is important for potential use of these phytochemicals as a new class of antibacterial agents in prevention and healing of honeybee colony against bacterial pathogens. However, experiments in vivo (using bees) would be necessary in order to explore the real potential of these compounds.

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Nouvelle substances anti-*Paenibacillus larvae* purifiées à partir de propolis

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Propolis de peuplier / agent anti-pathogène / pinocembrine / pinobanskine / acide 9-oxo-10(*E*)-12(*Z*) octodécadienoïque

Neue Anti-*Paenibacillus larvae*-Substanzen aus Propolis isoliert

Pappel / Propolis / Pinocembrin / 3-*O*-Acetyl-Pinobanskin / 9-oxo-10(*E*)-12(*Z*)-Octadecensäure / Anti-AFB-Substanzen

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