ACTA VET. BRNO 2018, 87: 277-284; https://doi.org/10.2754/avb201887030277

In vitro antibacterial activity of macelignan and corosolic acid against the bacterial bee pathogens *Paenibacillus larvae* and *Melissococcus plutonius*

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Received August 4, 2017
Accepted June 27, 2018

Abstract

Foulbrood disease, which is caused by *Paenibacillus larvae* (American foulbrood) or *Melissococcus plutonius* (European foulbrood disease), is a major threat to honeybees (*Apis mellifera*) worldwide. Tetracycline derivatives have been used to control these bacteria, but resistant strains have evolved, and the antibiotic derivatives can adversely affect bee health. When foulbrood disease is discovered, beekeepers usually burn the bee hives and equipment. The aim of this study was to investigate the *in vitro* susceptibility of *P. larvae* and *M. plutonius* to new antibacterial agents. Antibacterial activities of seven compounds prepared as serial two-fold dilutions were assayed using 96-well microtitre plates. Minimum inhibitory concentration values were obtained after 24 h or 48 h of incubation. Antibacterial synergistic activity of tetracycline and the test compounds was evaluated using broth micro-dilution assays with two-fold serial dilutions of the compounds. Among the seven compounds tested, macelignan and corosolic acid showed the strongest anti-bacterial activity. In addition, tetracycline interacted synergistically with corosolic acid to reduce *P. larvae* and *M. plutonius* growth. Even though macelignan and corosolic acid were worth as solely effective agents to treat *P. larvae* and *M. plutonius*, combinatorial treatment with tetracycline would be more useful to overcome toxicity, resistance occurrence and costliness. Further validation studies of these compounds and identification of their targets, as well as actual field tests and bee toxicity studies are still needed. However, macelignan and corosolic acid as natural secondary metabolites would be effective agents for bee foulbrood disease with valuable antibacterial activities.

Honeybee, infectious disease, natural compound, anti-bacterial agent

Approximately one third of the human food supply depends on insect pollination. Honeybees are the most economically important pollinators of agricultural and horticultural plants. Bee populations worldwide have been declining for various reasons (Genersch 2010; Cornman et al. 2012; Seitz et al. 2016). No single factor can account for the overall global decline, but a contributing factor is bacteria, including *Paenibacillus larvae* and *Melissococcus plutonius* (Miyagi et al. 2000; Alippi et al. 2002; Takamatsu et al. 2014; Takamatsu et al. 2016).

Paenibacillus larvae is a gram-positive endospore-forming bacterium known to cause American foulbrood (AFB) disease, which is one of the most economically devastating bee diseases. *Paenibacillus larvae* infects the brood stages of honeybees (*Apis mellifera* L.), and the infected honeybee brood turns black with a spotted appearance and bitter smell. The brood becomes a hard scale of material that sticks to the walls of the cells. Infected larvae usually die after the cell is sealed, and billions of infective spores form in their remains. *Paenibacillus larvae* spores remain viable for many years and are highly

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infectious and can spread to neighboring hives. They are very resistant to extremes of hot and cold and to many disinfectants. Some regions therefore have a "burn only" policy, but others allow the use of antibiotics to control the disease (Miyagi et al. 2000; Alippi et al. 2002; Genersch 2007; Ebeling et al. 2016).

Melissococcus plutonius is a gram-positive bacterium that causes European foulbrood (EFB) disease. European foulbrood is considered less virulent than AFB, but infected larvae usually die before they are capped (Bailey 1983; Forsgren et al. 2005; Takamatsu et al. 2014). Yearly reoccurrence of EFB from contaminated combs and equipment can occur. *Melissococcus plutonius* does not produce spores, but combs contaminated with the bacteria can still re-infect honey bees in subsequent years.

Foulbrood is considered a serious bee disease because it can cause massive damage to the apiculture industry (Forsgren 2010; Genersch 2010). The antibiotic oxytetracycline hydrochloride has been used to treat foulbrood (Thompson and Brown 2001; Thompson et al. 2005). However, continuous use of this antibiotic has reduced the viability of honeybees (Pettis et al. 2004; Thompson et al. 2005; Jasny 2017; Raymann et al. 2017), increased the chance of presence of antibiotics in honey (Mutinelli 2003), drove the evolution of antibiotic-resistant strains (Alippi et al. 2002; Evans 2003; Jasny 2017) and killed the valuable honey bee bacteria (Yoshiyama et al. 2009; Alberoni et al. 2016; Jasny 2017; Raymann et al. 2017). Those concerns have resulted in a decreased use of this antibiotic by honeybee keepers and have spurred scientists to find safer antibacterial agents that can be used to treat foulbrood.

Natural plants and medicinal herbs have been a valuable source of novel anti-microbial agents, because they have abundant biological activities and are pharmacologically safe (Albo et al. 2003; Özkırım et al. 2014; González et al. 2015; Shin and Kim 2016). In addition, frequent consumption of plants by humans suggests that many secondary plant metabolites are not toxic to humans (Park et al. 1999; Motsei et al. 2003; Webster et al. 2008; Maggi et al. 2011). We identified macelignan and corosolic acid as putative novel anti-fungal agents against *Ascosphaera apis* based on the screening of a natural compound library in a previous study (Shin and Kim 2016).

Here, we examined the antibacterial effects of macelignan and corosolic acid, as well as emodin-8-O-β-D-glucopyranoside, fangchinoline, loganic acid, tracheloside, dehydrocostus lactone, miconazole, congo red and kanamycine against the causative agents of foulbrood to identify new candidate compounds that have effective anti-bacterial activity against *P. larvae* and *M. plutonius*.

Materials and Methods

Strain maintenance

Glycerol stock of *P. larvae* (KCTC14031, Korea) was streaked on MYPGP (MYPGP: 1% Mueller-Hinton broth, 1.5% yeast extract, 0.2% glucose, 0.3% K_2HPO_4, and 0.1% sodium pyruvate) agar and cultured at 35 °C for 48 h. For the broth microdilution assay of *P. larvae*, Mueller Hinton broth with thiamine (MHBT) was used (Gende et al. 2008; CLSI document M100-S21 2009; Maggi et al. 2011). Glycerol stock of *M. plutonius* (ATCC35311, USA) was streaked on KSBHI medium (BHI medium plus 0.15 M KH_2PO_4 and 1% soluble starch) for 3 days at 35 °C in a 5% CO_2 atmosphere. For broth microdilution assay of *M. plutonius*, KSBHI broth medium was used (Takamatsu et al. 2014; Wu et al. 2014; Takamatsu et al. 2016). Glycerol stocks of other bacteria were streaked on Luria-Bertani (LB) agar (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl, and 4.5% (w/v) nutrient agar) and incubated aerobically at 37 °C overnight.

For *Staphylococcus aureus* (KCTC1621, Korea) and *Staphylococcus saprophyticus* (KACC15799, Korea), Mueller-Hinton broth (30.0% beef infusion, 1.75% casein hyrolysate, and 0.15% starch adjusted pH7) was used. For *Bacillus subtilis* subsp. *Spizizenii* (KCTC3705, Korea), *Escherichia coli* (KACC11598, Korea), *Micrococcus luteus* (KACC13377, Korea) and *Enterococcus faecalis* (KACC11304, Korea), Mueller-Hinton II broth was used.

In vitro antibacterial assay

Antibacterial activities of each compound were assayed based on the Clinical and Laboratory Standards Institute document (CLSI document M100-S21 2009) with slight modifications using 96-well microtitre plates (Miyagi

et al. 2000; Ansari et al. 2016; Vukic et al. 2017). Briefly, 10 mg/ml stock of each compound was prepared in dimethyl sulphoxide (DMSO). Test compounds (Chemface, Wuhan, China) and positive control miconazole (MB cell, Seoul, Korea), Congo red (DCC, Seoul, Korea) and kanamycine (Georgiachem, Georgia, USA), were prepared as serial two-fold dilutions with final concentrations ranging between 0.2–100 mg/l. A single colony of each bacterial isolate was separately inoculated into 3 ml of media. After that, each well was inoculated with each bacterial suspension at a final density of 0.5 McFarland. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that inhibited bacterial growth after 24 h or 48 h of incubation. Wells without any compounds were used as negative controls of growth. This experiment was repeated at least three times, and duplicate samples were prepared.

Antibacterial synergy testing

To assess if the test compounds and tetracycline had synergistic antibacterial activity, a broth microdilution assay was performed using a checkerboard design (Drogari-Apiranthitou et al. 2012; Shin and Kim 2016). Tetracycline was serially two-fold diluted, and test compounds were also vertically serially two-fold diluted and dispensed into a 96-well microtitre plate. One hundred microlitres of *P. larvae* or *M. plutonius* suspension at a density of 0.5 McFarland were placed in the wells. Final drug concentrations ranged from 10 mg/l to 0.01 mg/l tetracycline, 25 mg/l to 0.02 mg/l corosolic acid, and 12.5 mg/l to 0.1 mg/l macelignan. The MIC values of test compounds were individually determined on the same plate as the controls. Fractional inhibitory concentration indexes (FICIs) were used to assess if different combinations of compounds had synergistic effects. Tests were performed at least three times.

Results

Macelignan and corosolic acid showed antibacterial activity against *P. larvae* and *M. plutonius*

We tested the anti-*P. larvae* and anti-*M. plutonius* activity of seven compounds (loganic acid, macelignan, tracheloside, fangchinoline, corosolic acid, dehydrocostus lactone, and emodin-8-O-β-D-glucopyranoside) (Shin and Kim 2016). Of these seven compounds, macelignan had the strongest anti-*P. larvae* activity (MIC of 1.56 mg/l after a 24 h incubation and MIC of 3.125 mg/l after a 48 h incubation), and corosolic acid had comparable anti-*P. larvae* activity (MIC of 3.125 mg/l after 24 h and 48 h of incubation). Macelignan and corosolic acid also had high anti-*M. plutonius* activity (MIC of 3.125 mg/l at 24 h and 48 h incubation). The synthetic antifungal agents miconazole (1.56 ~ 3.125 mg/l) and tetracycline (0.39 ~ 1.56 mg/l) showed very high antibacterial activity against both strains, but loganic acid, tracheloside, fangchinoline, dehydrocostus lactone, emodin-8-O-β-D-glucopyranoside, kanamycin, and congo red showed very little or no anti-bacterial activity (Table 1).

Table 1. Susceptibility of *P. larvae* and *M. plutonius* to the tested compounds.

Compound	*P. larvae*		*M. plutonius*	
	24 h	48 h	24 h	48 h
Loganic acid	>200	>200	>200	>200
Macelignan	1.56	3.125	3.125	3.125
Tracheloside	>200	>200	>200	>200
Fangchinoline	50	>200	>200	>200
Corosolic acid	3.125	3.125	3.125	3.125
Dehydrocostus lactone	50	50	>200	>200
Emodin-8-O-β-D-glucopyranoside	>200	>200	>200	>200
Miconazole	1.56	3.125	3. 125	3. 125
Congo red	>200	>200	>200	>200
Kanamycin	>200	>200	>200	>200
Tetracycline	0.78	1.56	0.39	0.78

Minimum inhibitory concentration (MIC) values in mg/l

Next, we investigated if macelignan had antibacterial activity against a broad spectrum of human pathogens. Macelignan had anti-bacterial activity against *B. subtilis* subsp. *spizizenii, M. luteus, M. plutonius,* and *P. larvae* (MICs of 25 mg/l, 12.5 mg/l, 3.125 mg/l, and 3.125 mg/l after 48 h incubation, respectively) (Table 2). We also investigated if corosolic acid had broad spectrum antibacterial activity. Corosolic acid showed anti-bacterial activity against *M. luteus, M. plutonius*, and *S. saprophyticus* (6.25 mg/l, 3.125 mg/l, and 50 mg/l at 48 h, respectively; Table 3). In addition, tetracycline showed very high anti-bacterial activity against *B. subtilis* subsp. *spizizenii, E. coli, M. luteus, M. plutonius, P. larvae,* and *S. saprophyticus* (MIC values of 0.78 mg/l, 3.125 mg/l, 0.39 mg/l, 0.78 mg/l, 3.125 mg/l, and 3.125 mg/l after 48 h, respectively) (Table 4).

Corosolic acid and tetracycline showed synergistic anti-bacterial activity

We next investigated if tetracycline and macelignan or corosolic acid had synergistic suppressive effects against *P. larvae* and *M. plutonius* by performing broth microdilution assays using a checkerboard design (Tables 5, 6, 7, 8). Tetracycline did not interact synergistically with macelignan to reduce *P. larvae* and *M. plutonius* growth (FICI = 0.83 ± 0.14 and 0.67 ± 0.29, respectively). In contrast, tetracycline interacted synergistically with corosolic acid to reduce *P. larvae* and *M. plutonius* growth (FICI = 0.29 ± 0.07 and 0.41 ± 0.14, respectively).

Table 2. Susceptibility of various bacteria to macelignan.

Strain name		MIC	
		24 h	48 h
KCTC3705	*B. subtilis subsp. spizizenii*	1.56	25
KACC11598	*E. coli*	>200	>200
KACC11304	*E. faecalis*	>200	>200
KACC13377	*M. luteus*	12.5	12.5
ATCC35311	*M. plutonius*	3.125	3.125
KACC14031	*P. larvae*	1.56	3.125
KCTC1621	*S. aureus*	>200	>200
KACC15799	*S. saprophyticus*	6.25	>200

Minimum inhibitory concentration (MIC) values in mg/l

Table 3. Susceptibility of various bacteria to corosolic acid.

Strain name		MIC	
		24 h	48 h
KCTC3705	*B. subtilis subsp. spizizenii*	6.25	200
KACC11598	*E. coli*	>200	>200
KACC11304	*E. faecalis*	>200	>200
KACC13377	*M. luteus*	6.25	6.25
ATCC35311	*M. plutonius*	3.125	3.125
KACC14031	*P. larvae*	3.125	3.125
KCTC1621	*S. aureus*	>200	>200
KACC15799	*S. saprophyticus*	12.5	50

Minimum inhibitory concentration (MIC) values in mg/l

Table 4. Susceptibility of various bacteria to tetracycline.

	Strain name	MIC	
		24 h	48 h
KCTC3705	*B. subtilis subsp. spizizenii*	0.39	0.78
KACC11598	*E. coli*	3.125	3.125
KACC11304	*E. faecalis*	100	200
KACC13377	*M. luteus*	0.39	0.39
ATCC35311	*M. plutonius*	0.39	0.78
KACC14031	*P. larvae*	1.56	3.125
KCTC1621	*S. aureus*	200	200
KACC15799	*S. saprophyticus*	3.13	3.13

Minimum inhibitory concentration (MIC) values in mg/l

Table 5. Synergistic effects of macelignan and tetracycline against *P. larvae*.

P. larvae (P618)	MIC[1]				FICI[2]	
	Macelignan		Tetracycline		Macelignan + Tetracycline	
	24 h	48 h	24 h	48 h	24 h	48 h
Median	1.56	3.125	0.78	1.56	0.83 ± 0.14	0.83 ± 0.14

[1] Minimum inhibitory concentration (MIC) values in mg/l

[2] Fractional inhibitory concentration index (FICI)

Table 6. Synergistic effects of corosolic acid and tetracycline against *P. larvae*.

P. larvae (P618)	MIC[1]				FICI[2]	
	Corosolic acid		Tetracycline		Corosolic acid + Tetracycline	
	24 h	48 h	24 h	48 h	24 h	48 h
Median	3.13	3.13	0.78	0.78	0.29 ± 0.07	0.46 ± 0.07

[1] Minimum inhibitory concentration (MIC) values in mg/l

[2] Fractional inhibitory concentration index (FICI)

Table 7. Synergistic effects of macelignan and tetracycline against *M. plutonius*.

M. plutonius	MIC[1]				FICI[2]	
	Macelignan		Tetracycline		Macelignan + Tetracycline	
	24 h	48 h	24 h	48 h	24 h	48 h
Median	3.125	3.125	0.39	0.78	0.67 ± 0.29	1.0 ± 0

[1] Minimum inhibitory concentration (MIC) values in mg/l

[2] Fractional inhibitory concentration index (FICI)

Discussion

Tetracyclines are widely used as broad-spectrum antibiotics, but their use has decreased because of resistance development (Chopra and Roberts 2001). Oxytetracycline is a tetracycline derivative that has been used to treat infectious bacterial bee diseases such as

Table 8. Synergistic effects of corosolic acid and tetracycline against *M. plutonius*.

M. plutonius	MIC[1]				FICI[2]	
	Corosolic acid		Tetracycline		Corosolic acid + Tetracycline	
	24 h	48 h	24 h	48 h	24 h	48 h
Median	3.125	3.125	0.39	0.78	0.41 ± 0.14	0.33 ± 0.14

[1] Minimum inhibitory concentration (MIC) values in mg/l

[2] Fractional inhibitory concentration index (FICI)

AFB and EFB, but resistance has been reported (Alippi 1996; Miyagi et al. 2000; Evans 2003).

Commercially available antimicrobial compounds usually inhibit the growth of various microorganisms, including beneficial bacteria. If broad range antimicrobial agents such as tetracycline are used to treat pathogens, the growth of favourable organisms will also be affected. Moreover, there is a possibility of the presence of tetracyline in honey (Mutinelli 2003), which could cause the inhibition of favourable organisms in the consumer. Therefore, there is currently a focus on identifying narrow-range antimicrobial agents (Bax and Green 2015).

In this study, macelignan and corosolic acid showed the strongest anti-bacterial activity among the seven compounds we tested. In addition, macelignan and corosolic acid had narrow antimicrobial activity ranges. We previously reported that macelignan had antifungal activity by inhibiting fungal mitogen-activated protein kinase phosphorylation (Shin and Kim 2016). Moreover, macelignan showed antibacterial and sporicidal activity against *B. cereus* (Rukayadi et al. 2009). When macelignan was combined with 1,2-hexanediol, an alternative preservative in cosmetics without any side effect, a highly synergistic effect was observed as food-grade antimicrobials (Yogiara et al. 2015).

Corosolic acid is usually extracted from the banaba (*Lagerstroemia speciosa*) leaf. It is reported to exhibit antihyperlipidemic, antioxidant, antiinflammatory, antiviral, antineoplastic, osteoblastic, and protein kinase C inhibition activity (Kim et al. 2016). Several studies have reported its antimicrobial activity using plant extract or isolated corosolic acid, which showed similar activity compared with our results. In addition, no adverse effects have been observed or reported in animal studies or controlled human clinical trials (Stohs et al. 2013).

In our previous study (Shin and Kim 2016), macelignan and corosolic acid also showed antifungal activity, which suggests that macelignan and corosolic acid can be used to treat *A. apis* infections. Together, these results suggest that macelignan and corosolic acid show a potential for the treatment of bee-related infectious fungal and bacterial diseases.

Combinatorial treatment of *P. larvae* and *M. plutonius* with tetracycline and corosolic acid resulted in enhanced anti-bacterial activity compared to that of either compound alone. Combined treatment of bees with tetracycline and corosolic acid might therefore reduce the development of resistance and reduce costs. However, there is still a weak point because macelignan and corosolic acid could kill only vegetative stages, not spores. Thus, these substances should be combined with appropriate beehive disinfection such as heat treatments, use of disinfectants (Dobbelaere et al. 2001) or transfer of bees to clean beehives.

In conclusion, macelignan and corosolic acid showed a strong antibacterial activity against *P. larvae* and *M. plutonius* and a narrow spectrum of antibacterial activity against other bacteria. Corosolic acid and tetracycline had suppressive effects on bacterial growth. In a future study, we plan to determine the appropriate ratio of these two compounds to

maximize their anti-fungal and anti-bacterial activities and then perform a field test to confirm the effectiveness of these compounds.

As natural compounds, macelignan and corosolic acid should have minimal impact on the environment and are promising candidates for treating microbial bee diseases.

Conflict of Interest

This research was conducted in the absence of any commercial or financial relationships that could be construed as posing potential conflicts of interest.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A1A01061225) and the IPET (116094-03-1-SB010).

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