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Acaricidal Activity of Cyclodipeptides from *Bacillus amyloliquefaciens* W1 against *Tetranychus urticae*

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ABSTRACT:

Bioassay-guided fractionation of the supernatant of the biocontrol strain *Bacillus amyloliquefaciens* W1 led to the isolation of eight acaricidal cyclodipeptides from the active fractions by column chromatography separation and HPLC purification. The chemical structures of these compounds were identified as cyclo-(Gly-L-Phe), **2**, cyclo-(L-Phe-*trans*-4-OH-L-Pro), **3**, cyclo-(Gly-L-Tyr), **4**, cyclo-(L-Ala-L-Pro), **5**, cyclo-(L-Pro-*trans*-4-OH-L-Pro), **6**, cyclo-(Gly-L-Pro), **7**, cyclo-(L-Pro-L-Pro), **8**, and cyclo-(L-Tyr-*trans*-4-OH-L-Pro), **9**. Those cyclodipeptides displayed significant acaricidal activities with LC_{50} values of 13.85 – 98.24 μM. Cyclo-(L-Tyr-*trans*-4-OH-L-Pro) (LC_{50} 13.85 μM) was five times more effective than the positive control abamectin (LC_{50} 72.06 μM). The results indicated that the hydroxyl group is an important component. This is the first report on the acaricidal capabilities of cyclodipeptides against *Tetranychus urticae*. The results revealed that the acaricidal activity of the biocontrol strain *B. amyloliquefaciens* W1 was dependent on its constituent cyclodipeptides, which have the potential to be safe and environmentally friendly acaricides.

KEYWORDS:

Cyclic dipeptides; Diketopiperazine; Bioassay-guided fractionation; Two-spotted spider mite; Acaricidal activity; Biological control

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most polyphagous arthropod herbivores and feeds on more than 1,100 plant species belonging to more than 140 different plant families. It is a key pest in field crops and in greenhouses, devastating both annual and perennial crops.[1] The direct damage caused by the pest is owing to feeding punctures, leading to spotting of the leaves, which can significantly reduce the photosynthesis of plants. The chemical pesticides is major control method against *T. urticae* for long time. [2,3] The mites has been developing resistance to the most of chemical insecticides and acaricides owing to frequent and long term use of pesticides.[4] Additionally, chemical pesticides can have unintended consequences, impacting environmental quality, food safety, human health, and biodiversity including natural enemy abundance.[5]

Scientists have given much attention to biological control of spider mites, for example, the predators or parasites is widely used to regulate the miters, particularly the use of phytoseiid mites, which are currently sold worldwide.[6] Entomopathogenic fungi or entomogenous fungi such as *Hirsutella thompsonii* Fisher,[7] *Neozygites floridana* Weiser and Muma,[8] *Beauveria bassiana* (Balsamo) Vuillemin,[9] *Verticillium lecanii* (Zimm.),[10] and *Isaria cateniannulata*[11] also attract a large number of researchers' eyeballs. Few bacteria, however, have been reported as to their acaricide activities owing to the piercing-sucking mouthparts of the mites, which make it difficult for bacteria to infect them. In recent years, researchers have concentrated on

intracellular organisms such as *Wolbachia* that may cause distorted sex ratios in the mite offspring, thereby impacting populations,[12,13] and toxin-producing bacteria such as *Bacillus thuringiensis* that can produce crystal proteins called *δ*-endotoxins that are commonly used as a biological acaricides.[14] Additional research has been carried out on and potential acaricide producing strains such as *Pseudomonas putida*.[15]

Bacillus amyloliquefaciens W1 (CGMCC No. 11949), which was isolated from two-spotted spider mites that had died naturally, is a patented strain that has a strong capability to cause death of *T. urticae*.[16] The *B. amyloliquefaciens* W1 strain has been used for the prevention and treatment plant spider mites with a greater than 80% success rate in corn, strawberry and tomato greenhouses. However, its exact mechanism of action is not clear. In the present study, the secondary metabolites of *B. amyloliquefaciens* W1 were investigated for active compounds because the supernatant of this culture was found to have the highest acaricidal activity among the fermentation liquid, supernatant and cells of this strain.

MATERIALS AND METHODS

Microbial strain and culture conditions

B. amyloliquefaciens W1 was isolated from the two-spotted spider mite that had died naturally and was collected in Anning, Yunnan Province, China. It was identified based on the analysis of 16 S rDNA sequence.[16] The voucher specimen of W1 was preserved in the China General Microbiological Culture Collection Center (CGMCC No. 11949). The bacterium was inoculated into Luria-Bertani (LB) medium in an

Erlenmeyer flask and incubated for 72 h with 170 rpm at 35 °C and pH 8.

Plants and mites

The spider mite was collected from infested tomato plants in fields near the city of Anning (102° 19' E, 25° 01' N, 1835 m a.s.l.), Yunnan Province, China. It was not required specific permission for the collection because it is a harmful agricultural insect and is distributed extensively. We confirmed that the mites' collection did not impact endangered or protected wild.

The mites used in the research were feeding on tomato and corn in greenhouse that had been maintained for more than five years without the use of acaricides and pesticides.

Chemicals and reagents

All chemical reagents were of analytical purity or the highest one commercially available. The solvents ($CHCl_3$, MeOH, EtOH, EtOAc and Me_2CO) were bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, P.R. China), and were used directly without further purification. TFA, HCOOH, MeCN, and MeOH were purchased from Merck (Darmstadt, Germany) and used for HPLC. Abamectin was provided by Sigma (St Louis, MO), and L amino acid standards and Marfey's reagent FDAA were also purchased from Sigma. Analytical grade water was obtained by ultrafiltration.

Instrumentation

The NMR spectra were detected by DRX-500, DRX-600 and DRX-800 NMR spectroscopic instruments (Bruker, Bremerhaven, Germany) with TMS as an internal

92 standard. The data of MS were collected LCMS-IT-TOF apparatus (Shimadzu, Kyoto,
93 Japan). IR and UV spectra were measured on VECTOR22 FT-IR instrument (Bruker)
94 and UV-2450 UV-VIS spectrophotometer (Shimadzu, Japan) respectively. Column
95 chromatography was performed using a D101-type macroporous adsorption resin,
96 silica gel and Pre-coated TLC sheets of silica gel 60 GF254 (Qingdao Haiyang
97 Chemical, Inc. Qingdao, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB,
98 Sweden), and silica gel C18-RP (50 μm) (YMC, Tokyo, Japan). The TLC sheets spots
99 were observed with UV light instrument (Gongyi, Zhengzhou, China). A series 1200
100 HPLC equipped with an Eclipse XDB-C18 column 250 mm × 4.6 mm i.d., 5 μm, was
101 made use of compositional analysis, and an Agilent ZORBAX SB-C18 column 250
102 mm × 9.4 mm, 5 μm was applied to purify compound (Agilent, Santa Clara, CA). A
103 SZX10 stereomicroscope (Olympus Corp., Tokyo, Japan) was also used. Images were
104 captured with an AxioCam (Carl Zeiss, Thornwood, NY). A CR22G centrifuge
105 (Hitachi, Tokyo, Japan) was used to remove the bacteria cells from the fermentation
106 liquid.

107 **Extraction, isolation and purification**

108 The culture broth (10 L) of the *B. amyloliquefaciens* W1 strain was centrifuged for
109 15 min at 12000 rpm (14570 × g) to remove the bacteria cells. The supernatant was
110 absorbed by D101-type macroporous adsorption resin column chromatography (CC)
111 using 95% EtOH as the eluent to obtain a crude extract (29.6 g) after removing the
112 EtOH with a vacuum rotary evaporator at 55 °C. The extract was suspended in water

(400 mL, 50 °C), and then successively extracted with EtOAc (3 × 400 mL) and *n*-BuOH (3 × 400 mL) to obtain fraction E (2.2 g) and fraction B (5.9 g), respectively. Although the aqueous residue provided the majority of the weight from fraction W (21.2 g), this material had no effect on the spider mites. Fraction E was separated with silica gel CC using a gradient system of $CHCl_3$/EtOAc (20:1-1:1) as the eluent to obtain 9 fractions, E1-E9, in which only fractions E6 and E8 showed acaricidal activity. These two fractions were then analysed by preparative HPLC (mobile phase MeCN/H_2O, 40:60; UV 230 nm; the temperature of column 30 ºC; the velocity of flow 1 mL/min) to gained compound **1** (2.4 mg) from E6, compounds **2** (5.6 mg) and **3** (36.5 mg) from E8. Fraction B was severed into 12 fractions using C18 silica gel CC with a H_2O/MeOH gradient in which only B1, B2 and B3 had acaricidal activity. Fraction B1 was subjected to Sephadex LH-20 CC using methanol as the mobile phase to get compounds **4** (2.1 mg), **5** (1.5 mg), **6** (9.8 mg) and **7** (6.9 mg), which were further purified by preparative HPLC (mobile phase MeCN/H_2O, 55:45; UV 230 nm; the temperature of column 30 ºC; the velocity of flow 1 mL/min). Fraction B2 was purified with Sephadex LH-20 CC using methanol as the mobile phase to afford compounds **8** (2.6 mg), **9** (3.4 mg) and **10** (2.1 mg), which were further sublimated by HPLC (mobile phase MeCN/H_2O, 55:45; UV 230 nm; the temperature of column 30 ºC; the velocity of flow 1 mL/min).

The pure compounds was analyzed with TLC and analytical HPLC (mobile phase MeCN/H_2O, 55:45; injection volume 1 μL; UV 230 nm, the temperature of column 30

134 ºC; the velocity of flow 1 mL/min; run time 15 min).

135 **Absolute configuration evaluation of cyclodipeptides**

136 The experiment to determinate absolute configuration of compounds was carried
137 out according to the Marfey's method [17] with some modifications. Each cyclodipeptide
138 (1.5 mg) was mixed with 6 M HCl (1.0 mL), reacted for 24 h at 125 ºC. The products
139 were added in H_2O (100 μL), 1% FDAA acetone solution (200 μL) and 1 M $NaHCO_3$
140 (40 μL), and then heated for 1 h at 40 ºC with shaking. The reaction products was
141 quenched using 2 M HCl (20 μL). The mixture was allow to cool, dried, and dissolved
142 in MeOH for further analysis. Each kind of standard of L amino acid (1 mg) was used
143 to react with Marfey's regent in the same way. An Agilent 1200 series machine
144 equipped with an Agilent eclipse XDB-C18 column 250 mm × 4.6 mm i.d., 5 μm was
145 used for HPLC analysis at 30 ºC using two solvent systems: solvent A, 0.1% TFA
146 aqueous solution; solvent B, MeCN; linear gradient: 0 min, 25% B; 40 min, 60 % B;
147 45 min, 100 % B; UV 340 nm.

148 **Acaricidal activity assay**

149 The acaricidal activity assay, used to test the effects of contact toxicity against *T.*
150 *urticae* of *B. amyloliquefaciens* W1 and the different fractions of its secondary
151 metabolites, was composed of three methods, including the slide dip assay, the leaf
152 spray assay, and the microscale dip assay. The acaricidal activity components were
153 obtained from the fermentation liquid through bioassay-guided procedures using the
154 first two methods. The last method was carried out to evaluate the acaricidal ability of

the compounds that were purified from the acaricidal activity fractions.

Slide dip assay

The slide dip bioassay [18] (Figure 1) was used to assess the death rate of *T. urticae* treated with the isolations from the fermentation products of *B. amyloliquefaciens* W1. The different fractions dissolved in water containing 1% azone (to increase the compounds permeability in aqueous medium) were diluted to 0.5 mg/mL as test solutions. Adult mites were stuck to double-sided adhesive tape that was placed at the end of a glass slide (10 cm × 2 cm) using a brush. The energetic mites selected were stuck on the glass slide and took count of starting number under a stereomicroscope, and then the slide was dipped into the agent for 5 s, allowed to be upright until it dried using the absorbent paper to remove the agent, and observed under a stereomicroscope 2 h after treatment, similarly, 1% azone was served as the control. The experiment was carried out in triplicate. The dead mites' appendages will not move when brushed carefully.

Leaf spray assay

The acaricidal activity of all fractions, was similarly evaluated by the leaf spray assay [19] (Figure 1). Adult spider mites (50) were move to a fresh tomato leaf from the source leaves infested by *T. urticae*. They were then sprayed individually with various test agents that consisted of the different fractions of the fermentation products of *B. amyloliquefaciens* W1, which were dissolved in water containing 1% azone and diluted to 0.5 mg/mL. The leaves were placed onto cotton soaked with water to make the leaf

alive for 2 h, and then observed under a stereomicroscope. Water with 1% azone used as the control. The experiment was performed in triplicate. The dead mites' appendages will not move when brushed carefully. The half leaf spray assay [20] was also used to detect the acaricidal activity, with the same procedures as in the leaf spray assay. One half leaf was sprayed with test solution, and the other half was sprayed with water in 1% azone to serve as the control. The Abbot's formula[21] was used to calculate the corrected mortality rate (%).

Microscale dip assay

The microscale dip assay [22] (Figure 1), designed especially for microcomponents, was used to evaluate the acaricidal ability of the compounds isolated from the acaricidal activity fractions. The compounds were dissolved in water containing 1% azone and were diluted gradually to 50.00 μg/mL, 25.00 μg/mL, 12.50 μg/mL, 6.26 μg/mL, 3.13 μg/mL, 1.56 μg/mL, and 0.78 μg/mL as test solutions. Adult mites, moved from the leaves to smooth white paper using a soft brush, were affixed to a needle pinpoint with adhesive and inspected under a stereomicroscope to take away any dead or immobile one, and maintain only the active one as the starting number. The needle was immersed into the compound solutions (contained in a centrifuge tube) for 5 s, and then observed and counted under a stereomicroscope 2 h after treatment. Similarly, 1% azone aqueous used as the control. The experiment was triplicate. The dead mites' appendages will not move when brushed carefully.

Statistical analysis

197 The mortalities were corrected using Abbot's formula [21].

$$Pt = [(Po - Pc)/(100 - Pc)] \times 100$$

198 Where *Pt* represents the means of corrected mortality (%), *Po* represents the means of
199 the treated mortality (%) and *Pc* represents the means of the control mortality (%).

200 Data analysis was done using descriptive statistics software MINITAB version 14.
201 Concentration-mortality data were conducted to probit analysis for compounds lethal
202 concentation (LC_{50}) and toxicity regression equation. The LC_{50} values for each
203 acaricidal preparation and theit treatments were consdered to be significantly different
204 from one another when their 95% confidence limits did not overlap.

205 **RESULTS AND DISCUSSION**

206 **Identification of compounds**

207 Ten compounds were obtained from the culture broth of *B. amyloliquefaciens* W1
208 by bioassay-guided fractionation for acaricidal activity, with CC separation and HPLC
209 purification, and conducted to various spectroscopic analyses, such as NMR and MS.
210 The structures identified for eight compounds **2**–**9** were classified as diketopiperazines
211 or cyclodipeptides, as shown in Figure 2, and an additional two compounds
212 corresponded to macrolactin A, **1** and bacimethrin, **10**. by analysing and comparing for
213 the NMR spectra with that reported in the literature, they were identified as the
214 following: macrolactin A, **1**,[23] cyclo-(Gly-L-Phe), **2**,[24] cyclo-(L-Phe-*trans*-4-OH-L-Pro),
215 **3**,[25] cyclo-(Gly-L-Tyr), **4**,[26] cyclo-(L-Ala-L-Pro), **5**,[27] cyclo-(L-Pro-*trans*-4-OH-L-Pro),
216 **6**,[28] cyclo-(Gly-L-Pro), **7**,[29] cyclo-(L-Pro-L-Pro), **8**,[27] cyclo-(L-Tyr-*trans*-4-OH-L-Pro),

9,[26] and bacimethrin, **10**.[30] All compounds purities was greater than 95% according to the peak area in HPLC analysis.

Analysis of Marfey's derivatives of cyclodipeptides

The absolute stereochemistry of the eight cyclodipeptides were determined with the modified Marfey's method. The results showed that eight cyclodipeptides contain L amino acids, apart from the glycine, which is achiral.

Acaricidal activity for bioassay-guided fractionation

The acaricidal activity components were isolated from *B. amyloliquefaciens* W1 through bioassay-guided fractionation. The corrected mortality rate of *T. urticae* treated with each fraction was recorded (Figure 3). The culture broth, supernatant and its extract showed effects on the spider mites, and the extract was then subjected to chemical analysis to produce different the fractions. Fractions E6, E8, B1, B2 and B3, which were derived from activity fractions E and B, were more effective (corrected mortality rate ranging from 86.0% – 93.0% for slide dip assay and 86.0% – 91.2% for the leaf spray assay) than the others, including the positive control abamectin (corrected mortality rate of 80.7% for the slide dip assay and 78.9% for the leaf spray assay). All of the tested fraction concentrations were standardized at 0.5 mg/mL in aqueous solution containing 1% azone, except for the culture broth, supernatant and strain samples.

Acaricidal activity of pure compounds

All isolates **1**–**10** were evaluated for acaricidal activity with a microscale dip assay

against *T. urticae* and were compared with the positive control abamectin under the same conditions. Among the compounds, the cyclodipeptides **2**–**9** exhibited a potent lethality, with LC_{50} values ranging from 13.85 – 98.24 μM (Table 1), which is similar to or higher than the activity of abamectin (LC_{50} 72.06 μM). Macrolactin A, **1** and bacimethrin, **10** displayed only a weak activity (data not shown). The LC_{50} values of cyclodipeptides **2**–**9** against *T. urticae* were 84.60, 25.19, 43.98, 98.24, 43.71, 95.96, 63.90 and 13.85 μM, respectively, with the isolated compound **9** (LC_{50} 13.85 μM) being approximately five times more effective than the positive control (LC_{50} 72.06 μM). These results indicate that cyclodipeptides **2**–**9**, particularly cyclo-(L-Tyr-*trans*-4-OH-L-Pro), **9**, have potential for the development of new acaricidal agents for the control of *T. urticae*.

Structure-activity relationship

Natural peptides are well known as a kind of significant resources of medicinal key composition. Among all cyclic peptides, the cyclodipeptide is the simplest one, which compose a great family of metabolites biosynthesized by bacteria, fungi, flora, and fauna. They display extensive bioactivities, for instance bioherbicide, algicidal, antibacterial, antimicrobial, anticancer, antitumour, antiviral, antifungal, and antihyperglycaemic activities.[31-36] In this study, eight cyclodipeptides were isolated from *B. amyloliquefaciens* W1 and were tested for acaricidal agents against the two-spotted spider mite. The isolated cyclodipeptides exhibited distinctive acaricidal activities against *T. urticae*, with LC_{50} values of 13.85 – 98.24 μM. We demonstrated

259 that the acaricidal efficacy of *B. amyloliquefaciens* W1 was dependent on the various
260 cyclic dipeptide components. Furthermore, this is the first report on the acaricidal
261 capabilities of cyclodipeptides against *T. urticae*.

262 Interestingly, the acaricidal activities of the diketopiperazines change with a
263 change in the amino acid, and amino acids with hydroxyl groups, such as
264 hydroxyproline and tyrosine, could greatly improve the activity. A comparison of the
265 structure (Figure 2) and LC_{50} (Table 1) data of the diketopiperazines reveals that
266 compounds that are cyclized by two amino acids without a hydroxyl group, such as
267 cyclo-(Gly-L-Phe), **2**, cyclo-(L-Ala-L-Pro), **5**, cyclo-(L-Gly-L-Pro), **7**, and
268 cyclo-(L-Pro-L-Pro), **8**, have lower acaricidal activities than other diketopiperazines
269 with hydroxyl group, such as cyclo-(D-Phe-*trans*-4-OH-D-Pro), **3**, cyclo-(Gly-Tyr), **4**,
270 cyclo-(L-Pro-*trans*-4-OH-L-Pro), **6**, and cyclo-(L-Tyr-*trans*-4-OH-L-Pro), **9**. The
271 compound **9** with two hydroxyl groups which is cyclized by tyrosine and
272 hydroxyproline demonstrated the best acaricidal activity among eight isolates.
273 Therefore, the hydroxyl groups in diketopiperazines are important for acaricidal
274 capability.

275 In agricultural and horticultural cultivation, various chemical acaricides have been
276 greatly used to control spider mites because of their outstanding trait, such as having a
277 wide selection, quick mode of action and low cost. However, the frequent application
278 of chemical pesticides has led to various issues, such as pesticide resistance, pesticide
279 residues, concerns regarding food safety and human health, and impacts on

biodiversity, including natural enemy abundance.[37] However, some diketopiperazines have acaricidal capabilities against *T. urticae*, are safe and eco-friendly, and many of them have been reported to have other bioactivities or are used as medicinal components. For example, cyclo-(L-Pro-Gly), **7** can enhance memory.[38] Cyclo-(L-Pro-L-Pro), **8** can be used for chemical selectivity oxidations of sulfides.[39] Cyclo-(Gly-Tyr), **4** has been shown to inhibit bioluminescence and exhibit synergistic antimicrobial activity and has also been used as herbicide.[40-42] Therefore, the isolated diketopiperazines **2**–**9**, and in particular, compound, **9** cyclo-(L-Tyr-*trans*-4-OH-L-Pro), have the potential to be safe and environmentally friendly acaricides.

Although the resurgence of spider mites, induced by chemical pesticides, is the result of a combination of several factors from the homeostatic modulation of the pest itself to the reduction of natural enemies, pesticide resistance is thought to contribute to the resurgence phenomenon.[43] *T. urticae* quickly progresses to acaricide resistance, generally only a few years after the implementation of novel acaricides. Some acaricides such as clofentezine, abamectin and hexythiazox used in greenhouses to control *T. urticae* on roses and other economic crops, have lost most of their impact.[44] The *T. urticae* resistance have been arose to nearly one hundred ingredients of acaricides.[45] Therefore, the problem has become urgent, as *T. urticae* is presently deemed as the most resistant species as far as the sum total of compounds to which populations have turned resistant.[2] It is significant to effectively manage insecticide resistance to preserve the usefulness of insecticide whenever, and we have many

options such as cultural methods, autocidal techniques, crop rotation, semiochemicals, host plant resistance, chemical control, genetically modified plants, and biocontrol.[46] In this study, we used the biocontrol strain *B. amyloliquefaciens* W1 to control *T. urticae* in the greenhouse, which is in accordance with insecticide resistance management procedure. Ten natural products were obtained from the strain, of which eight compounds displayed acaricidal activity against *T. urticae*. Natural compounds have been used effectively as biocontrol agents not only due to their environmental safety, and ease of degradation but also because they do not elicit drug resistance. Our findings help to provide an alternative source of microbial acaricides that do not have the unwanted effects of drug resistance, residual agrochemicals and environmental pollution.

ASSOCIATED CONTENT

Supporting Information

This supporting information is available free of charge via the Internet at http://pubs.acs.org.

Figures S1−S30: ^{1}H NMR spectrum, ^{13}C NMR and DEPT spectrum, and ESI-MS spectrum of compounds; Figures S31-38: HPLC profile of FDAA derivatives of amino acid standards and compounds; Figure S39: HPLC chromatogram of compounds; Figure S40: The follow chart of compounds from the supernatant of *B. amyloliquefaciens* W1; Physicochemical parameters of compounds.

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Notes

The authors declare no competing financial interests.

ABBREVIATIONS USED

HPLC, high performance liquid chromatography; CC, column chromatography; TLC, thin layer chromatography; LB, Luria-Bertani; TFA, trifluoroacetic acid; FDAA, 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; MS, Mass spectrometry; LCMS-IT-TOF, liquid chromatography-mass spectrometry-ion-trap-time of flight instrument; EtOH, ethanol; EtOAc, ethyl acetate; BuOH, butanol; MeCN, acetonitrile; MeOH, methanol; Me_2CO, acetone; LC_{50}, half maximal inhibitory concentration.

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492 **FIGURE CAPTIONS**

493 Figure 1. Scheme of acaricidal activity assay methods. (A): slide dip assay; (B): leaf
494 spray assay; (C): microscale dip assay

495 Figure 2. The structures of compounds **1**–**10**

496 Figure 3. Corrected mortality rate of *T. urticae* treated with culture broth, bacteria cells,
497 supernatant, 0.5 mg/mL fractions from the supernatant, and 0.5 mg/mL abamectin in
498 the slide dip assays and leaf spray assays. Bars represent standard deviations of means.
499 (A): slide dip assays; (B): leaf spray assays.

Table 1. LC_{50} Values of Isolated Compounds **2**–**9** and Abamectin against *T. urticae*.

Compound	Regression equation [b]	LC_{50} [b] (μM)	95% Confidence interval [b]	R	SE
2	y = 2.31x + 0.55	84.60	62.45 − 114.63	0.96	0.05
3	y = 2.35x + 1.70	25.19	17.54 − 36.18	0.99	0.02
4	y = 3.19x − 0.24	43.98	34.26 − 56.46	0.99	0.02
5	y = 2.15x + 0.72	98.24	71.54 − 134.90	0.98	0.05
6	y = 2.76x + 0.48	43.71	32.91 − 58.05	0.95	0.03
7	y = 2.29x + 0.46	95.96	71.59 − 128.61	0.98	0.04
8	y = 2.30x + 0.85	63.90	47.93 − 85.19	0.95	0.04
9	y = 1.99x + 2.72	13.85	7.80 − 24.59	0.99	0.02
PC[a]	y = 2.51x + 1.86	72.06	52.43 − 99.05	0.99	0.07

[a] positive control: abamectin

[b] The regression equation, LC_{50} and 95% confidence limit were determined by probit analysis.

Figure 1

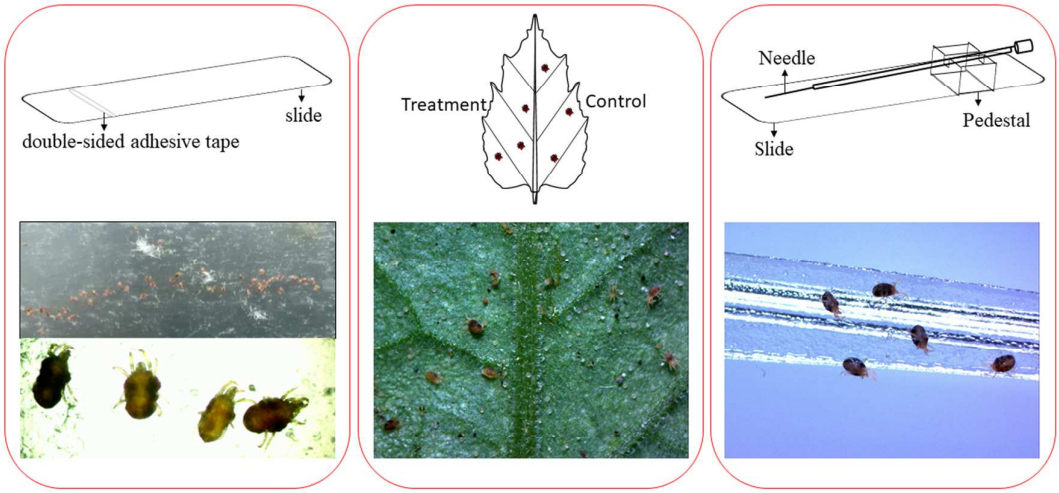

slide
double-sided adhesive tape
Treatment
Control
Needle
Pedestal
Slide
A
B
C

Figure 2

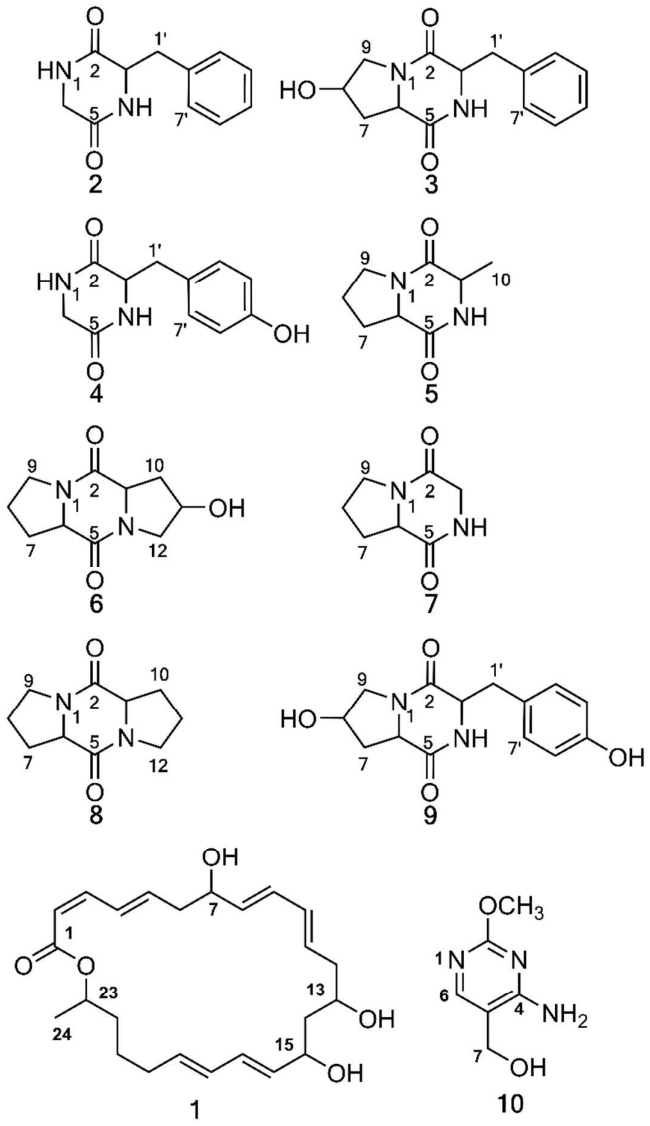
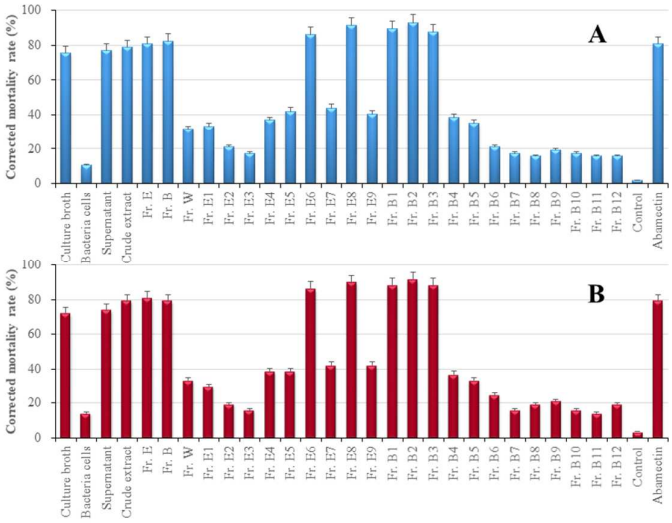


Figure 3


A
B
Corrected mortality rate (%)
0
20
40
60
80
100
Culture broth
Bacteria cells
Supernatant
Crude extract
Fr. E
Fr. B
Fr. W
Fr. E1
Fr. E2
Fr. E3
Fr. E4
Fr. E5
Fr. E6
Fr. E7
Fr. E8
Fr. E9
Fr. B1
Fr. B2
Fr. B3
Fr. B4
Fr. B5
Fr. B6
Fr. B7
Fr. B8
Fr. B9
Fr. B10
Fr. B11
Fr. B12
Control
Abamectin

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