Insecticidal Potency of a New Bioactive Dipeptide from *Bacillus velezensis* AR1 Culture Supernatant Against *Myzus persicae*

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Abstract

The green peach aphid (*Myzus persicae*) has become a worldwide challenge causing high yield losses through direct plant attack and transmission of plant pathogens. A widespread resistance development against synthetic agrochemicals by the aphid and the negative impact of the chemicals used to manage the pest on human health, other organisms and the environment are other problems. Therefore, the aphicidal potency of the secondary metabolite from *Bacillus velezensis* AR1 was investigated under in-vitro conditions. The purification process was conducted using solid-phase extraction by Strata SI-1 column, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Then, the purified EA3T3 fraction was analysed with the nuclear magnetic resonance (NMR ^{1}H, ^{13}C, H-H COSY, HSQC, and HMBC) and the metabolite was identified as 5-N-tyrosinylornithine. The aphicidal potency of the metabolite on *Myzus persicae* mortality was tested using three concentrations over 24 to 72h periods of exposure in a laboratory bioassay in comparison with a biopesticide E-II. The biopesticide EII caused the highest mortality of the aphids; 89% at 72 hr. The purified metabolite caused a mortality rate of 40.74±6 to 66.67±5% at 1 to 4µg mL^{-1} concentrations after 72h of exposure. The aphid mortality increased with the increase in concentration and duration of exposure to EA3T3. The lowest LD_{50} was attained at 72h of exposure. Therefore, the bioactive metabolite produced by *B. velezensis* AR1 is potent against the green peach aphid *Myzus persica* and has the potential to be used for the management of aphids.

Keywords: Aphicide, biopesticide, 5-N-tyrosinylornithine

Introduction

The green peach aphid (*Myzus persicae*) is considered one of the most important pest species and attacks more than 800 plant species worldwide (Van Emden and Harrington, 2007). The aphid is economically important because adults and nymphs suck plant juices and transmit about 100 different diseases such as potato leafroll, beet western yellows, potato virus, pepper mottle virus, and bean mosaic (Berry and Mooney, 1998; Tharp *et al.*, 2005; Atsebeha *et al.*, 2009). Aphids also damage plants by injecting toxins from their saliva that cause curled leaves and malformations (Tharp *et al.*, 2005). In Ethiopia for instance, devastating effects of aphids on wheat and barley (Chemeda, 2015), and pepper (Atsebeha *et al.*, 2009) had been reported. The most common method of aphid control involves the application of soil insecticides before plant emergence followed by foliar applications during the growing season (Berry and Mooney, 1998). The application of pesticides, irrespective of their type, has been increasing worldwide to increase productivity. In contrast, the chemicals have negative impacts on the environment, in general, and on human health, in particular (Chapin, 2000; Parmesan and Yohe, 2003; Schwinges *et al.*, 2019). Furthermore, the intensive use of insecticides over many years has led to the development of resistance in *M. persicae* (Silva *et al.*, 2012; Bass *et al.*, 2014; Fray *et al.*, 2014; Panini *et al.*, 2014). An Invitro experiment in Ethiopia also indicated development of resistance by the cotton aphids population to various insecticides (Shonga *et al.*, 2008).

Thus, to overcome problems associated with the extensive use of chemical insecticides, alternative approaches, mainly, integrated pest management (IPM) such as the use of metabolites produced by biological control

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agents have been widely studied in many countries (Verbon and Liberman, 2016; Khasa, 2017; Regassa *et al.,* 2018).

Bacillus thuringiensis is the most widely used soil-dwelling microbe which produces vegetative insecticidal proteins (Vip3), an important tool for crop protection against caterpillar pests in IPM strategies (Banyuls *et al.,* 2018). In addition, certain *Bacillus* species such as *Bacillus amyloliquefaciens,* (Yun *et al.,* 2013; López-Isasmendi *et al.,* 2019) and combined fermentation culture of *Bacillus* sp. strain with *Pseudomonas* sp. (Naeem *et al.,* 2018) enhanced the production of active aphicidal metabolites that could control *M. persicae.* Furthermore, metabolites from *Bacillus cereus*, *B. amyloliquefaciens*, and *Bacillus subtilis* reduced aphid population and its effect on plant (Herman *et al.,* 2008; Gadhave *et al.,* 2016). Although there are limited reports available in Ethiopia, integrated use of entomopathogenic fungi with insecticide showed promising results in aphids control (Chemeda, 2015).

Bacillus species has been known for the synthesis of bioactive amino acids peptides and fatty acids that have insecticidal property to protect the plant from pests (Kumar *et al.,* 2014; Banyuls *et al.,* 2018;Regassa *et al.,* 2020). The current work also investigated the efficacy of the purified and identified active dipeptide compound from n-butanol extract of *B. velezensis* AR1 culture broth (Bayisa *et al.,* 2020) against green peach aphid. The isolate AR1 used in this work has been characterized for its antifungal activity, mainly by producing chitinase and 1.3,β-glucanase, as well as an antifungal mono methyl succinate (Regassa *et al.,* 2018; Regassa *et al.,* 2020). Therefore, this research investigated the aphicidal potency of the active secondary metabolites against *M. persicae*.

Materials and Methods

Bacterial culture broth fermentation and crude extract

Tryptic soy broth (TSB) growth media was autoclaved at 121 °C for 20 min and this culture broth was inoculated with overnight-grown AR1 cells. The inoculated media was fermented for ten days at 30 °C and 120 rpm in a shacking incubator. Following centrifugation at 4 °C and 6000 rpm for 15 min, the cell-free supernatant of the isolate AR1 was acidified with 0.1 N HCl to pH 3.0 and then extracted with an equal volume of n-butanol. The soluble organic fraction was concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) to obtain the crude extract.

Extraction and purification of bioactive metabolite

Approximately 70 g n-butanol extract concentrate, obtained through the method mentioned above was dissolved in a small amount of methanol, and subjected to silica gel column chromatography (Kieselgel 60, 70–230 mesh, Merk, Darmstadt, Germany) with stepwise elution by CH_3Cl: MeOH (100:0, 97.5:2.5, 90:10, 80:20, 70:30, 40:60, 50:50 and 0:100; v/v). All the eluted fractions were concentrated in vacuo (EYELA rotary vacuum evaporator) to yield a semisolid mass. Fractions were dissolved in MeOH and subjected to high-performance liquid chromatography (HPLC) fitted with a PrepHT C18 column (7 × 300 mm, 10 μm inner diameter), following sterile syringe (syringe size 0.45 μm; Hyundai micro, Seoul, Korea) filtering to obtain clear peaks. The elution was monitored by using an SPD-10 UV–visible detector (Shimadzu, Kyoto, Japan) at 210 and 254 nm wavelengths with a manual injection. Acetonitrile and water (60:40), at a flow rate of 1.75 mL min^{-1} was used as a mobile phase. Purification of the compound (500 mg) was done by solid phase extraction using Strata SI-1 (55u, 70A) column employing gradient of CH_3Cl and ethyl acetate (5:0, 4.95:0.05, 4.90:0.1, 4:1, 3: 2, 0:5) solvent system. The active fraction eluted with 4.9:0.1 solvent system was concentrated in vacuo and spotted (1 μL) on TLC plate (aluminum silica gel 60F254, TLC; Machery-Nagel, Duren, Germany), and the plate was run in a beaker containing $CHCl_3$: MeOH: EtA (7:2:1) for further purification. The bands on the TLC plates were visualized under 254 nm UV light (Vilber Lourmat, Collégien, France). Using the same solvent system, 260 mg of the compound was separated by PLC silica gel

60F2541 mm (glass plate 20 × 20 cm, Germany). Fraction collected from the prep-TLC plate was dissolved in HPLC-grade methanol analyzed by HPLC using an analytical C18 column (2 µL, 4.6 × 250 mm), UV detector at 254 nm and acetonitrile: water (60:40) as a mobile phase at a flow rate of 1.75 mL min^{-1}. The compound detected as a single peak was labelled as EA3T3.

Identification of the purified bioactive metabolite

The structure of the secondary metabolite was determined using Nuclear Magnetic Resonance spectroscopy (NMR ^{1} H, ^{13}C, H-H COSY, HSQC and HMBC). Five milligrams of the purified compound was dissolved in 0.75 mL methanol-D_4 (CD_3OD) and then subjected to spectral analysis. The NMR spectra were recorded using a DRX 500 NMR instrument (Bruker, Rheinstetten, Germany) operating at 600 MHz for ^{1} H and 125 MHz for ^{13}C at room temperature. Chemical shifts were reported in ppm (δ) by using heavy water (D_2O) and deuterated chloroform ($CDCl_3$) as a solvent and tetra-methyl-silane $(CH_3)_4Si$ as an internal standard.

Rearing of green peach aphid

The stock population was obtained from the insect molecular phylogeny and ecology laboratory at the Department of Applied Biology, College of Agriculture and Life Sciences, Chonnam National University, Republic of Korea. The aphids were subsequently reared on tobacco plants (*Nicotiana tabacum*) at 27°C and relative humidity of 50 to 60% in a sanitized insectarium.

Aphicidal activity of the purified bioactive secondary metabolite

The insecticidal activity of the secondary metabolite, EA3T3, was tested against the aphid. The second instar aphids were selected by a magnifying hand lens. Ten second-instar, aphids, which are at a high feeding stage**,** were placed on a 4*4 cm fresh cabbage leaf cut and placed in an insect culture plate (cat no. 310102, dish style 100*40 mm d*h, ventilation hole size 40mm, mesh hole size 0.053mm, and hole position is cap). Stock solution was prepared by dissolving 1 mg of concentrated secondary metabolite EA3T3 in 1 ml 50% ethyl acetate and 50% methanol. Treatments were then prepared from the stock solution as 0, 1, 2, 4 µg mL^{-1} and the biopesticide E-II, (commercial bio-formulated pesticide, extracted from *Sophora flavescens* obtained from Green focus co. Ltd. Republic of Korea), was used as a comparative control at 1000 dilution (as per the company recommendation). Blank 1 ml 50% ethyl acetate and 50% methanol solvent was used as control. Exactly 1 mL of each treatment was sprayed per plate using plastic white 2 mL sprayer bottle following the topical application method (Paramasivam and Selvi, 2017), on the uncovered leaf surface on which aphids were placed. All assays were conducted in triplicates. Mortality data were recorded at 24, 48, and 72h posttreatment. The percent reduction of the aphid population was corrected using the Abbott formula (Fleming and Retnakaran, 1985). Toxicity symptoms caused by EA3T3 fraction and EII-based biopesticide, such as shrinkage or dehydration that led the pest to necrosis as well as color changes on the outer cuticle of the tested aphids were apparent under a low power stereo microscope. Externally observable effects on the aphids treated with the metabolites and the biopesticide E-II were also digitally photographed.

Statistical analysis

The mortality data were analyzed by GLM using SAS Software 9.4 (SAS Institute, 2012). Mean comparisons were performed using protected least significant difference ($P < 0.05$) t-test. The lethal dose (LD_{50}) was computed by SAS software using the probit model.

Results

Extraction and purification of the bioactive secondary metabolite

At each chromatography step, impurities were removed. The 9:1 $CHCl_3$: MeOH fraction obtained from BuOH extract was subjected to Strata SI-1 solid phase extraction column and eluted with chloroform and ethyl acetate solvent gradient and six fractions were obtained, and depending on its antifungal activity the third fraction was further purified by preparative TLC.

Six bands on the Prep-TLC plate were separated and analyzed by HPLC. Fraction 3 (EA3T3) was detected as a single peak at 8.88min retention time (Fig. 1).

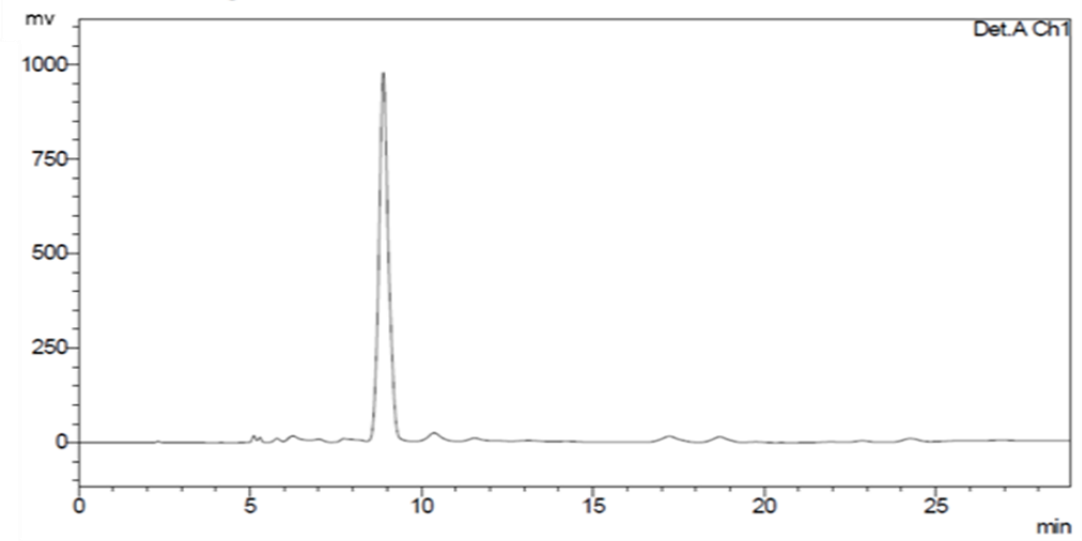

Figure 1. HPLC spectrum of EA3T3

Structural determination of the purified compound

The ^{1} H NMR spectrum (600 MHz, CD_3OD) of EA3T3 showed p-substituted benzene ring protons at δ 7.04 (2H, d, J = 9.0 Hz, H-2 and H-6) and 6.71 (2H, d, J = 9.0 Hz, H-3 and H-5), two nitrogen (N)-bearing methine proton signals at δ 4.37 (1H, dt, J = 4.8, 1.8 Hz, H-8) and 4.05 (1H, ddd, J = 11.4, 6.0, 2.4 Hz, H-2'), and one N-bearing methylene proton signals at δ 3.53–3.57 (2H, m, H-5'a) and 3.34–3.38 (1H, m, H-5'b) (Table 1).

Table 1. ^{1}H and ^{13}C NMR data of EA3T3 in CD_3OD

Position	δ_H (*Int.*, *Multi.*, *J* in Hz)	δ_C (Position)
1	-	127.7
2, 6	7.04 (2H, d, 9.0)	132.3
3, 5	6.71 (2H, d, 9.0)	116.3
4	-	157.8
7a	3.10 (1H, 13.8, 4.8)	37.9
7b	3.00 (1H, 13.8, 4.8)	
8	4.37 (1H, dt, 4.8, 1.8)	58
9	-	167.2
1'	-	171
2'	4.05 (1H, ddd, 11.4, 6.0, 2.4)	60.2
3'a	2.07-2.11 (1H, m)	22.8
3b	1.17-1.24 (1H, m)	
4'	1.78-1.83 (2H, m)	29.5
5'a	3.53-3.57 (1H, m)	46.1
5'b	3.34-3.38 (1H, m)	

NMR data (δ) were measured at 600MHz for ^{1}H and at 125MHz for ^{13}C in CD_3OD, respectively. Proton coupling constants (*J*) in Hz are given in parentheses. The assignments were based on ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

Additionally, three methylene proton signals were observed at δ 3.10 (1H, m, J = 13.80, 4.8 Hz, H-7a), 3.00 (1H, m, J = 13.80, 4.8 Hz, H-7b), 2.07–2.11 (1H, m, H-3'a), 1.17–1.24 (1H, m, H-3'b) and 1.78–1.83 (2H, m, H-4') (Fig. 2A). These proton signals were confirmed by the ^{13}C NMR and HSQC spectra.

The ^{13}C-NMR (150 MHz, CD_3OD) spectrum showed 14 carbon (C) signals, including two carbonyl Cs at δ 167.2 (C-9) and 171.0 (C-10) (Fig. 2B). The presence of tyrosine was established by ^{1} H - ^{1} H COSY correlations (Bayisa *et al.*, 2021) of H-2/H-3 and H-7/H-8 and HMBC correlations [Fig. 2(c), arrows] of δ 3.10 (H-7a) and 3.00 (H-7b) to 167.2 (C-9).

Additionally, the presence of ornithine was assigned by ^{1} H - ^{1} H COSY correlations of H-2'/H-3', H-3'/H-4', and H-4'/H-5' and HMBC correlations of δ 2.07–2.11 (H-3'a) and 1.17–1.24 (H-3'b) to 171.0 (C-1'). In particular, the correlation of δ 3.53–3.57 (H-5'a) and 3.34–3.38 (H-5'b) to δ 167.2 (C-9) was observed in the HMBC experiment, indicating that the C-9 of tyrosine was amidified with the C-5 of ornithine. Therefore, the planar structure of EA3T3 was determined as 5-N-tysinosylornithine [Fig. 2(c)].

Aphicidal activity of the Purified organic compound

A total of four increasing concentration gradients of the fraction EA3T3 with one biopesticide (E II) as a control were employed. The purified dipeptide compound caused higher mortality which increase with increase in concentration and duration of exposure (Table 1). The biopesticide (1000x dilution) E-II, applied as a comparative control caused the highest aphid mortality at all exposure periods. The commercial biopesticide could cause about 89% aphid mortality within three days. Similarly, the mortality of the aphid increased with the increase in concentration of purified dipeptide along with the period of incubation. The three concentrations of the purified organic compound showed significant ($p< 0.05$) differences at all exposure periods. The highest aphid population mortality was recorded for 4 µg mL^{-1} followed by 2 µg mL^{-1}. However, there was no significant ($p> 0.05$) difference between 1 and 2 µg mL^{-1} treatments in all exposure periods (Table 2). On the other hand, there was no significant ($p> 0.05$) difference between E-II and 4 µg mL^{-1} concentration at 24 and 48h exposure periods.

Lethal Dose of the purified organic compound

The time to kill the aphid was related to the concentration of the organic aphicide. About 9.19±3 µg mL^{-1} is required to control the pest by 50% within 24h (Fig. 3). Similarly, the estimated amount of the compound needed to cause the same mortality is about 3.03 and 2.35 µg mL^{-1} at 48 and 72h, respectively.

Toxic effect of the organic compound on green peach aphid cuticle

In addition to the study how organic compound caused aphid mortality, observation was made on its toxic effect on the outer cuticle of the pest. Observed changes include abrasive effects that caused shrinkage or dehydration that led the pest to necrosis as well as color changes on the body. Clear body shrinkage was observed on the cuticle of those aphids exposed to 4 µg mL^{-1} and E-II at 72h. Similarly, after death, the aphid's cuticle turned dark around their abdomen which evidenced the toxicity of the organic compound at 2, 4 µg mL^{-1} and E-II at all exposure periods (Fig. 4).

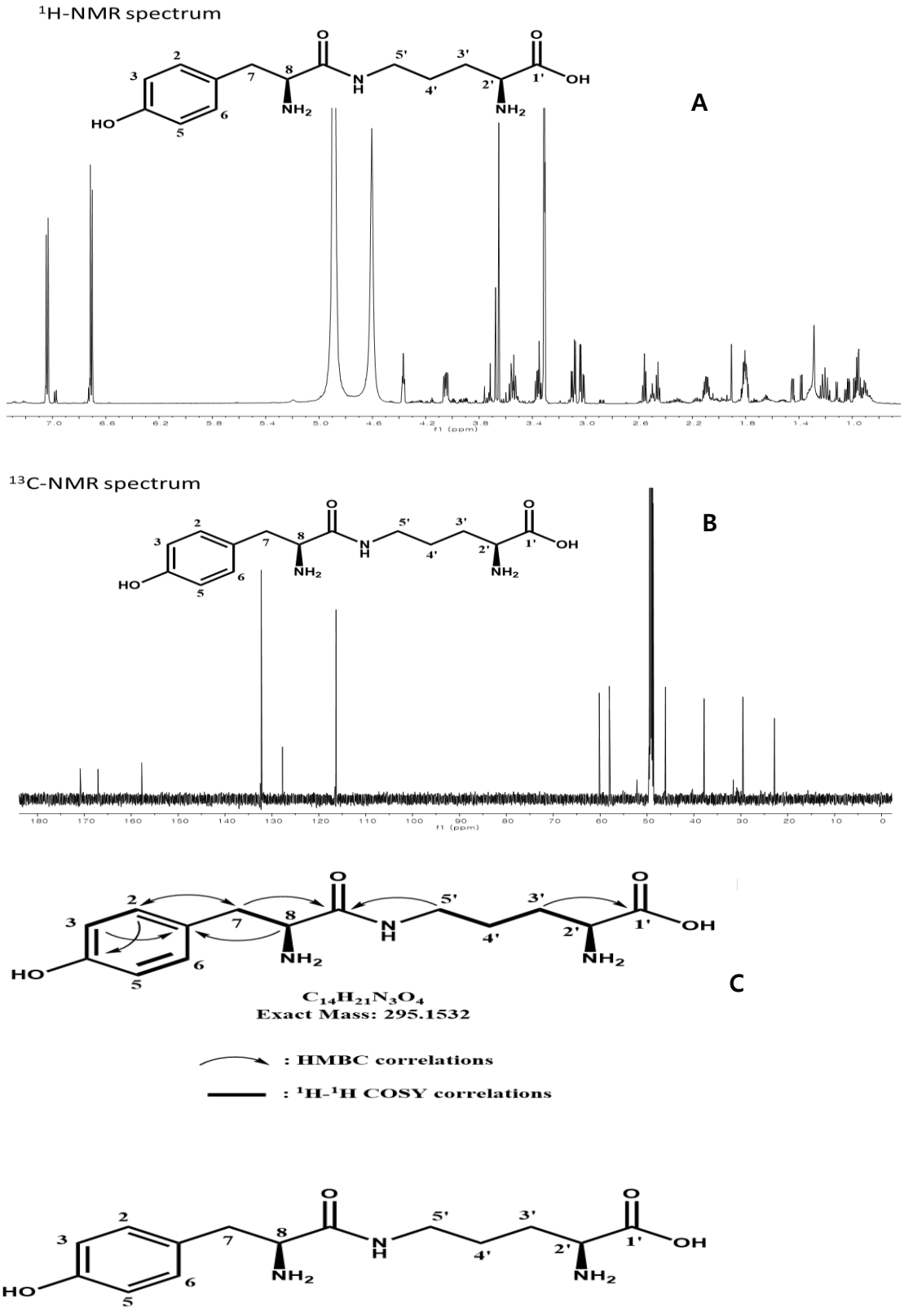

Figure 2. Structural identification of the purified active secondary metabolite. The ^{1}H-NMR spectrum (A), ^{13}C-NMR spectrum (B), and HMBC and 1H–1H COSY correlation (C) structure of the purified organic compound from the culture supernatant of *Bacillus velezensis* AR1.

Table 2. Effects of the secondary metabolite on green peach aphid mortality.

Treatments	Mean Aphid population mortality (%) (±*SE*)		
	24h	48h	72h
E-II	31.06±7.45a	67.85±5.05a	88.89±5.24a
1µgEAT3	10.38±2.81b	24.97±0.00b	40.74±6.05c
2µgEAT3	10.38±2.81b	39.26±2.92b	48.15±3.02bc
4µgEAT3	27.61±4.87ab	57.13±5.05a	66.67±5.24b

Data presented means ±SE (n=3). Columns followed by the same letter are statistically not different from each other at 5% confidence level.

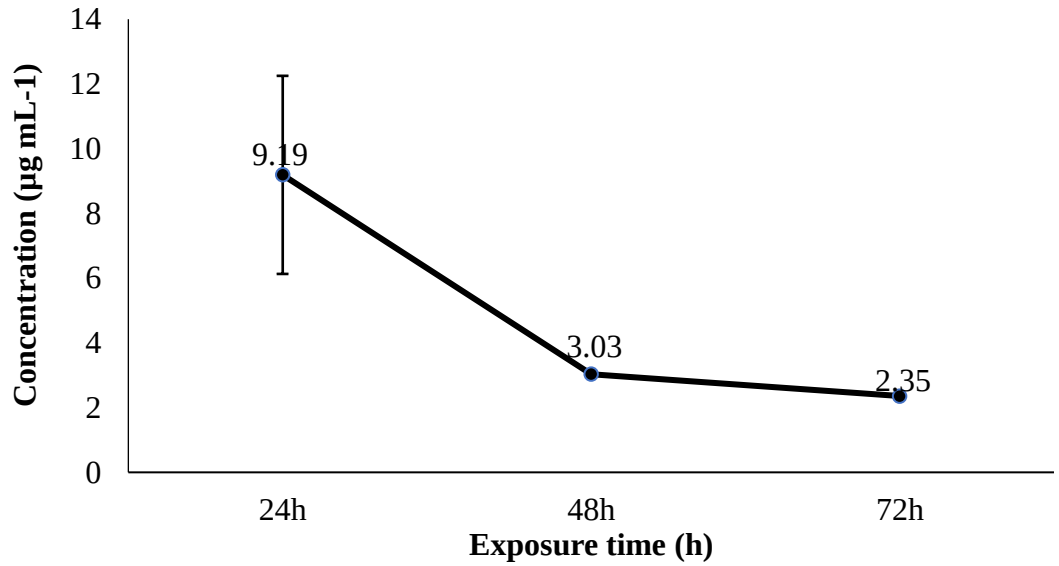

Figure 3. Lethal dose (LD_{50}) of the organic compound **EAT3**. The LD_{50} was determined by SAS probit model and E-II values were not included. Error bars denote the standard error of the mean (n=3).

Discussion

The purified organic compound investigated in this work has recently been reported for its strong antifungal activity (Bayisa *et al.*, 2021). *Bacillus velezensis* produce secondary metabolites and extracellular enzymes. For instance, *B. velezensis* AR1 produced monomethyl succinate in the culture broth extracted with ethyl acetate (Regassa *et al.,* 2020) and catalytic chitinase and 1.3 β-glucanase enzymes (Regassa *et al.*, 2018). Strain AR1 demonstrated strong antifungal activity and induced systemic resistance to *Sesamum indicum* (sesame) against *Alternaria sesami* (Bayisa, 2020). This species is well-known for its special gene responsible for about ten secondary metabolite clusters (Palazzini *et al.,* 2016). Other recent reports have also revealed that lipopeptide compounds, such as surfactin, iturin, fengycin and bacillomycin L produced by *B. velezensis* that suppressed various plant pests (Lim *et al.,* 2017; Cao *et al.,* 2018).

Several dipeptide secondary metabolites have been isolated from varying species of bacteria that have antimicrobial activities. For instance, several cyclic dipeptides antifungal compounds were isolated from *B. cereus* subsp. *Thuringiensis* (Kumar *et al.,* 2014); and from *Lactobacillus plantarum* (Kwak *et al.*, 2014). In recent works, several

Vip3 vegetative insecticidal amino acid proteins from *B. thuringiensis* (Banyuls *et al.*, 2018) and other plant entomotoxic proteins and *B. thuringiensis* endotoxins (Carlini and Grossi-de-Sá, 2002) were identified and reported. However, this is the first report on the isolation of 5-N-tyrosinylornithine from *B. velezensis* that was tested against *M. persicae*.

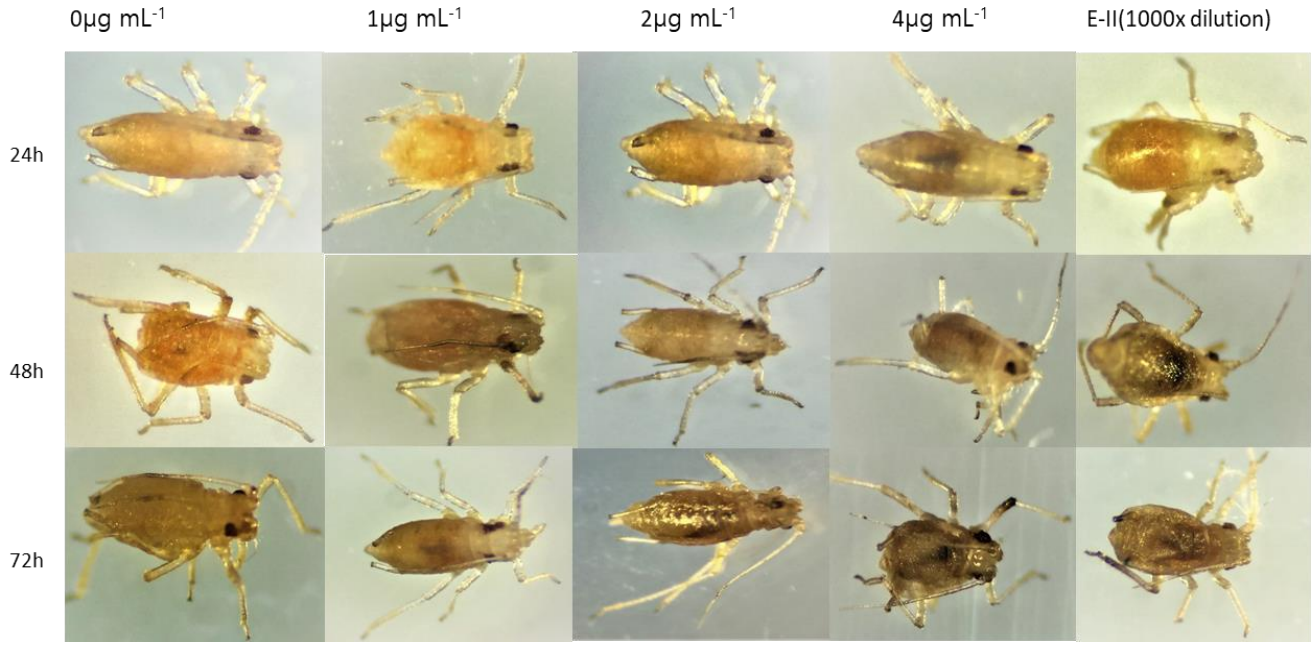

Figure 4. Toxicity effect of the secondary metabolite causing changes on aphid cuticle. The adult aphids were placed on cabbage leaf and sprayed with 1ml of the treatment and kept at 25° C. data were periodically collected using microscope.

A recent report indicated that three strains of *Bacillus amyloliquefaciens* CBMDDrag3, PGPBacCA2, and CBMDLO3, and their metabolites caused 100% green peach aphid mortality (López-Isasmendi *et al.*, 2019). In the present work, the purified secondary metabolite caused high mortality of 66.67±5% on *M. persicae* at 4µg mL^{-1} concentration after 72h exposure period. This signifies that the metabolite has the potential to replace toxic insecticides to control *M. persicae* and other aphids. Besides aphids, metabolites from different species of Bacillus are toxic to many insects. *Bacillus amyloliquefaciens* AG1 produces a lipopeptide biosurfactant exhibiting an LC_{50} of about 180 ng/cm^2 against *Tuta absoluta* larvae (Khedher *et al.*, 2015). Similarly, a biosurfactant produced by *B. subtilis* SPB1 against third instar larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) had LC_{50} of 257.0 mg/g six days post treatment (Ghribi *et al.*, 2012). In the current report, the metabolite produced by the *B velezensis* AR1 caused LC_{50} at 9.19, 3.03 and 2.35 µg mL^{-1} after 24, 48 and 72h, respectively. This implies that the secondary metabolite activity becomes more toxic to the insect with the extended periods of exposure. The biopesticide 5-N-tyrosinylornithine showed toxic symptoms in the green peach aphid. Similarly, Mohamad *et al.* (2013) reported muscular contractions around abdominal spiracles, sporadical leg twitching, dehydration and cuticle color changes due to the toxic effect of the lauric acid-based natural pesticide.

Conclusion and Recommendations

In addition to the most popular *B. thuringiensis* producing Vip3 protein, genes and secondary metabolite which has been used as biocontrol for insects, *B. velezensis* has now emerged as a potent bioinsecticide producing agent. *Bacillus velezensis* AR1 could produce enzymes including chitinase, protease, β-1,3-glucanase etc., and many hormones as well as other secondary metabolites that could cause cellular lysis and fatal damage to insect protein and cell wall. The bioinsecticide involved in the current work caused high mortality of up to 67 % and only 2.35 µg mL^{-1} of the metabolite could have caused LC_{50} after three days on green peach aphid. Therefore, the bioactive metabolites produced by *B. velezensis* AR1 have pesticide properties and the potential to be used against the green peach aphid and other aphids.

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