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Antibacterial activity of larval extract from the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) against plant pathogens

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Abstract
The antibacterial activity of the extracts of whole black soldier fly larvae (*Hermetia illucens*) was evaluated and was tested against six strains of plant pathogens. The extracts were prepared by homogenization after mixing grinded larvae with 0.01% acetic acid at 4 °C for 12 hr. Two methods, agar well diffusion and growth curve assay, were used to study the antibacterial activity against these pathogens. In our study, the antibacterial properties of the extracts were demonstrated by growth inhibition of all six tested bacterial pathogens. Inhibition zone assay and fluorescence assay confirmed that larval extracts have significant antibacterial activity against bacterial pathogens. The data provide further evidence that larval extracts play a role in the defense against microorganisms.

Keywords: Black soldier fly, Larvae, Antibacterial activity, Plant pathogens, *Hermetia illucens*

1. Introduction
It has long been documented that insects form the most diverse group of organisms on earth [13]. These large numbers of insect species and individuals are explained by several factors, including their remarkable reproductive abilities, the capability of flight, and their general adaptive abilities to the environment [13]. In *Drosophila*, antimicrobial peptides were found in the epithelium of different organ systems which may come in contact with the environment, such as the respiratory, digestive, and reproductive tracts, the malpighian tubes, and the mouthparts [17]. In the medical field, flies have been reported to reduce or eliminate bacteria in infected wounds [7] through either ingestion and digestion of the bacteria or generation of antimicrobial compounds. Similarly, earthworms (*Eisenia foetida*) reduced Salmonella enterica serovar *Enteritidis* populations in horse manure [10]. Antimicrobial peptides are known to be the most important elements of the insect immune system, providing high resistance to a wide range of pathogens and parasites [2]. Insects have been shown to be effective against bacterial and fungal plant pathogens [6]. In recent years, several reports have described the presence of two fractions with antibacterial activity, present either in the whole body or in the excreta/secreta of the maggots [5]. One fraction has a molecular weight (MW) of 2−10 kDa, and the other of <1 kDa [1]. An antibacterial substance, p-hydroxycinnamaldehyde, isolated from the larvae of the saw fly *Acantholyda parki* S. was also found to have a broad antibacterial spectrum against both gram-negative and gram-positive bacteria [9]. The black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) (BSF) is considered a beneficial insect. The larvae have been proposed as an application to reduce livestock manure [14], as they significantly diminish accumulated dry matter in poultry [14] and dairy manure [11]. BSF larvae reduce pathogen loads in manure; Erickson et al. [3] determined that they can suppress O157:H7 and serotype Enteritidis (ME 18) populations in poultry manure [3]. They also examined the ability of BSF larvae to reduce these pathogens in dairy cow and hog manure. Based on these findings, similar antibacterial substances might be present in BSF larvae. Hence in the present study an attempt was carried out to elucidate the antibacterial activities of extracts from whole BSF larvae.

2. Materials and methods
2.1 Sample preparation
BSF larvae extract was obtained by the method described below. 1500 larvae were washed 3 times with sterilized water and stored at -80 °C. The freeze-dried larvae (100 g) were

homogenized before addition of 800 mL of 0.01 % acetic acid. To remove the solid residues, the mixture was stirred at 4 °C for 12 hours and filtered with filter paper. The mixture was centrifuged at 13,000 × g for 20 minutes to separate the solution layer containing the larval extract. The separated layer was then filtered through a sterilized 0.45 μm membrane filter (Advantec MFS, Dublin, CA, USA). The sample was stored at -20 °C until later use. All experiments were done in 2013 (April-September).

2.2 Used strains and media

The bacterial strains used in this study, including 10466 (*Pseudomonas marginalis pv. marginalis*), 11153 (*Xanthomonas campestris pv. vesicatoria*), 10391 (*Pseudomonas viridiflava*), 11132 (*Pseudomonas sp.*), 12130 (*Pseudomonas syringae pv. tabaci*), 12965 (*Xanthomonas campestris pv. vesicatoria*), 10701 (*Ralstonia solanacearum*), and 11151 (*Xanthomonas axonopodis pv. glycines*), were obtained from the Korean Agricultural Culture Center (KACC). The strains were placed in a prepared glycerol stock (40 %, v/v) and stored at -80 °C. Bacteria to be used in this study were cultured in LBA, NA, and TSA (Oxoid) media. As for the bacterial culture, a colony from each solid medium was inoculated into 20 mL sterilized LB, NB, and TSB liquid media. After 12 hours of shaking culture in an incubator, 10 μL of cultured bacteria were taken and injected into 10 mL liquid media. The mixture was stirred until an absorbance range of 0.39–0.40 was achieved at 600 nm.

2.3 Extraction and fractionation

Ultrafiltration was performed to investigate differences in the antibacterial activity according to the molecular weight of the larval extract. An ultrafiltration cell (Milipore, Ireland) was used for the ultrafiltration, and 30, 10, and 3 kDa ultrafiltration membranes were used to perform Micro Weight Cut-off (MWCO) according to molecular weight. The extracted fractions were purified using a sterilized 0.45 μm membrane filter and then stored at -20 °C in the order of <3 kDa, 3–10 kDa, 10 –30 kDa, and >30 kDa for an agar diffusion assay.

2.4 Antibacterial activity measurement

2.4.1 Inhibition zone assay

Twenty milliliters of culture media were cooled to 45 °C, and 20 μL of bacteria with an OD (Optical density) of 0.39–0.40 at 600 nm were inoculated onto the culture media. In this study, an agar well diffusion assay was used to measure the antibacterial activity. The bacterial culture solution was gently mixed and poured into an 85 mm petri dish. After the media had solidified, 4 mm-wells were punched into the solidified medium and the sample was poured into the wells as a way of dispersing it throughout the medium. 0.01% acetic acid was used instead of the extract as a control for each bacterial species. The petri dish was incubated at the optimal culture temperature for each bacterium for 24 hours, and the diameter of bacterial growth inhibition was measured as a result. The experiments were performed in triplicates and mean values were obtained. In order to investigate the antibacterial activity of the larval extract, 10 μL of bacterial solution with an OD of 0.39–0.40 at 600 nm was inoculated into 10 mL of nutrient media and larval extract were added. The mixture was cultured in a 150 rpm rotary shaker. Absorbance was measured at 600 nm using a UV-spectrophotometer, and bacterial growth was determined every 2 hours up to 16 hours.

2.4.2 Fluorescence assay

LIVE /DEAD® BacLight. The Bacterial Viability Kit (Molecular probes, Life technologies, OR, USA) was used to investigate the antibacterial activity of the larval extract. Larval extract (250 mg) were added to 10 mL of the quality control bacteria with an OD of 0.39–0.40 at 600 nm, left for 12 hours, then centrifuged (10,000 rpm, 5 minutes, 4°C) and washed twice with 0.85 % NaCl solution. For the LIVE/DEAD® BacLight™ assay, 6 μL SYTO-9 and 6 μL PI were diluted with 2 mL of dH_2O, and 100 μL of diluted reagent was added to 100 μL of each bacterial solution. The mixture was reacted in the dark at room temperature for 30 minutes and then observed with a fluorescence microscope.

2.4.3 MIC (Minimum Inhibitory Concentration)

The larvae extract was diluted to concentrations of 50, 100, 150, 200, and 250 mg/mL, and 50 μL of larval extract at each concentration were placed on 4 mm-wells followed by culturing at the respective optimal temperature for 24 hours. The diameter of the zone of inhibition was measured in mm and the result was recorded. A zone of inhibition less than 7 mm in diameter was interpreted as no antibacterial activity.

3. Results and discussion

3.1 Inhibition zone assay and optical density

Two antibacterial analyses were performed to confirm the antibacterial activity of BSF larval extract. An agar diffusion assay was performed to analyze how larval extract react with the experimental strains. BSF larval extract demonstrated antimicrobial activity against 8 plant pathogenic strains that were tested (Fig. 1). The mean zone of inhibition was 12.2–25.0 mm.

Fig. 2 shows the results of the antimicrobial activity treatment of 3 out of 8 strains over time. The control exhibited a lag phase for 8 hours, and then its OD values rapidly increased. Nonetheless, the OD values of the larvae extract treatment groups showed almost no increase or an extremely small increase after 8 hours (Fig. 2). BSF larval extract were confirmed to have clear antibacterial activity against plant pathogenic bacteria, but there was a difference in the ability to inhibit bacterial growth depending on the type of bacteria.

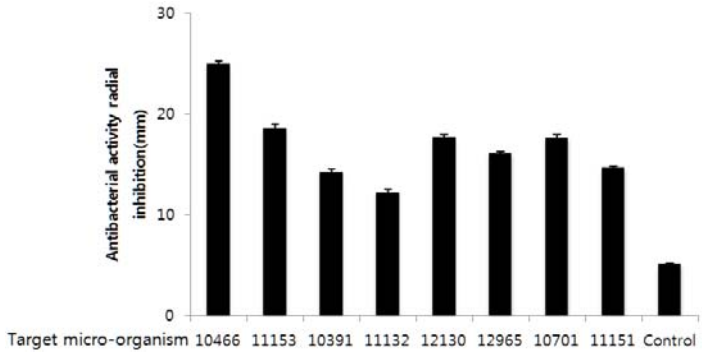

Fig 1: Antibacterial activity of *H. illucens* extracts against plant pathogens. 8mm filter paper, 100 μl loading, control: 0.01% acetic acid

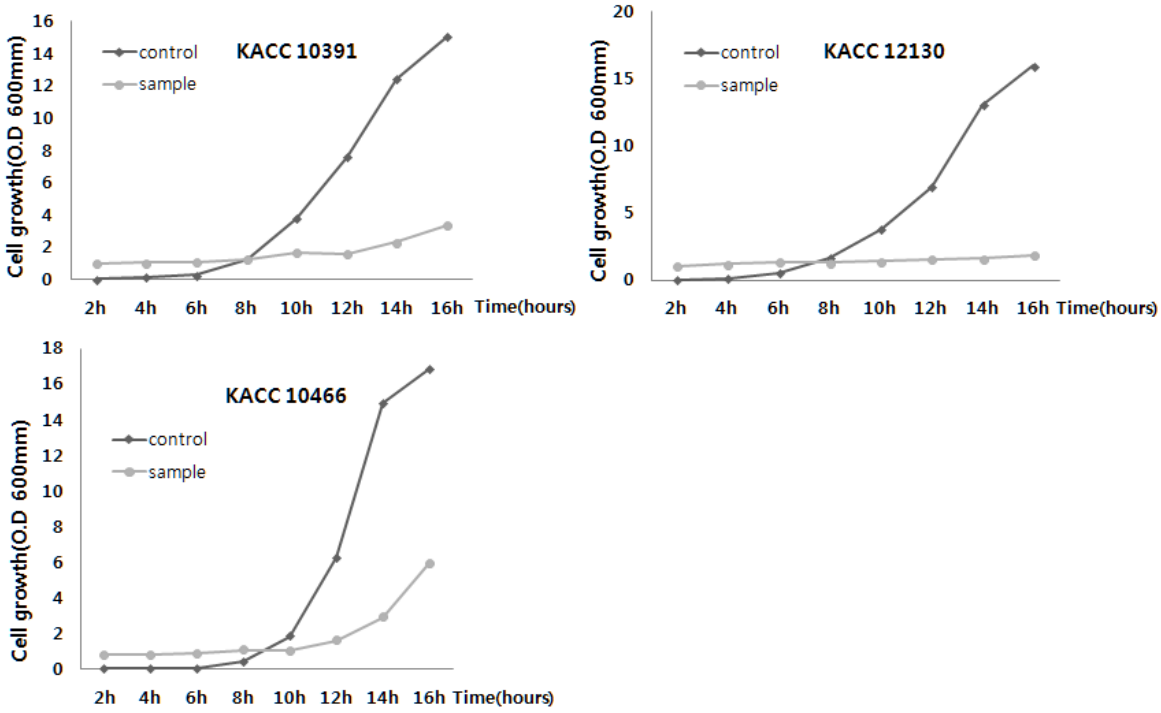

Fig 2: Effect of *H. illucens* larval extracts against plant pathogens.

3.2 Minimum inhibition concentration (MIC) of larval extract

Table 1 shows the MIC of the experimental bacteria. MIC refers to the minimum concentration of extract necessary for inhibition of microorganism growth. As described in the table, 5 different concentrations (50, 100, 150, 200, 250 µg/mL) were obtained from the extract. The results showed that the MIC of *Pseudomonas marginalis* (KACC10466), *Pseudomonas viridiflava* (KACC10391), and *Pseudomonas syringae* (KACC12130) were 50 mg/mL, 100 mg/mL, and 150 mg/mL, respectively.

Table 1: Minimum inhibitory concentration (MIC) of *H. illucens* larval extracts against plant pathogens. Degree of clarity of inhibition zone by growth inhibition, activity is classified as inactive (X) or active (O).

Strain	concentration of extracts(mg ml-¹)					
	50	100	150	200	250	control
KACC10391	X	O	O	O	O	X
KACC10466	O	O	O	O	O	X
KACC12130	X	X	O	O	O	X

3.3 Fluorescence assay

Live/Dead cell staining was performed to observe cell viability (Fig. 3). SYTO-9 is membrane-permeable, and passes through all cell membranes to label nucleic acids with green fluorescence. On the other hand, PI is membrane impermeable, and stains the nucleic acids of bacteria with damaged cell membranes to emit red fluorescence [15]. When stained with the SYTO-9 and PI fluorescent agents, viable bacteria are labelled green by SYTO-9, and dead bacteria are red by PI. As shown in the figure, cells in the control without larvae extract treatment were viable. Although small green regions were detected in the experimental groups, the cells were stained red overall, which indicates that the larvae extract has an effect on cell death.

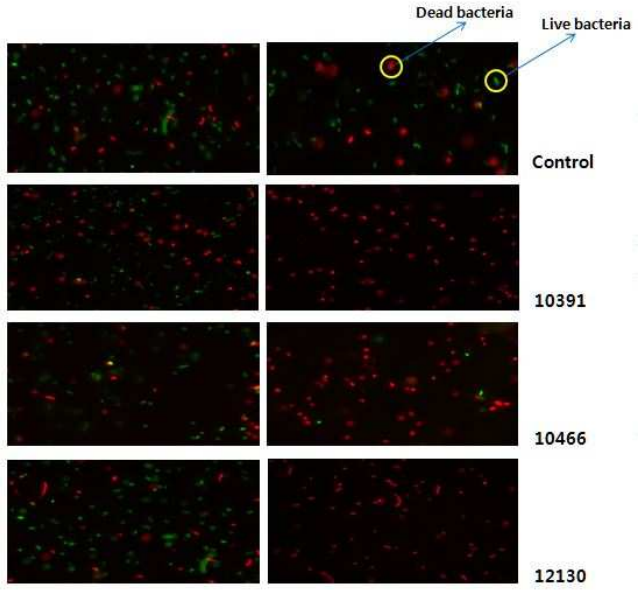

Fig 3: Investigation into bacterial viability. Live bacteria are green, dead bacteria are red. Control: Non-treated larval extraction.

3.4 Extract fractionation and antibacterial activity

In order to identify antibacterial agents from BSF larvae extract, the extracts were subjected to ultrafiltration (UF) using an UF cell (3 kDa, 10 kDa, 30 kDa cut-off) and substances with the molecular weight of <3 kDa, 3–10 kDa, 10–30 kDa, and >30 kDa were separated to assess their antibacterial activities (Table 2). Regarding the antibacterial activity of low molecular weight substances under 3 kDa that passed through the UF, the size of the clear zones for *Pseudomonas marginalis, Pseudomonas viridiflava,* and *Pseudomonas syringae* were 4.2 mm, 14.6 mm, and 17.8 mm, respectively, while for substances with a molecular weight of 10–30 kDa, the clear zones were 25.6 mm, 15.1 mm, and 19.8 mm. In particular, the highest activity was detected for substances

between 3–10 kDa, with clear zones of 31.2 mm, 22.6 mm, and 23.9 mm, respectively. Therefore, the antibacterial substances in larvae extract are believed to consist of 3–10 kDa substances. Antibacterial peptides are reported to play diverse roles in the host defense systems that exist in almost all living organisms in nature [16, 12, 8], with more than 500 antibacterial peptides discovered to date. These are reported to be mostly alkaline peptides comprised of 18–50 amino acids, with a molecular weight of 2–6 kDa and carrying a positive charge. Owing to their properties as effective antibiotics that can control numerous resistant bacteria due to the nature of their action, it is anticipated that antibacterial peptides could be used as novel biological immune activators [4].

Table 2: Activity of larval extract ultrafiltration fractions from black soldier fly against plant pathogens. 50ul/well, 250mg/ml (0.01% Acetic acid)

Ultrafilteration fraction	Activity(inhibition mm)		
	Antibacterial		
	KACC10466	KACC10391	KACC12130
>30kDa	17.05	-	-
10-30kDa	25.65	15.13	19.85
3-10kDa	**31.23**	**22.61**	**23.92**
<3kDa	24.22	14.6	17.84
Control	-	-	-

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