



## RESEARCH PAPER

**Detection of antimicrobial substances from larvae of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae)**Soon-Ik PARK<sup>1</sup>, Byung Soo CHANG<sup>2</sup> and Sung Moon YOY<sup>1</sup><sup>1</sup> Department of Biological Sciences, Dankook University, Cheonan, Korea<sup>2</sup> Department of Cosmetology, Hanseo University, Seosan, Korea**Correspondence**

Sung Moon Yoe, Department of Biological Sciences, Dankook University, Dandae-ro, Dongnam-gu, Cheonan-si, Choongcheongnamdo, 330-714, Korea.  
Email: smyoe@dankook.ac.kr

Received 3 January 2014;  
accepted 3 February 2014.

doi: 10.1111/1748-5967.12050

**Abstract**

Maggots have become highly successful in the treatment of non-healing wounds and multidrug-resistant pathogen infections. The main objective of this study was to extract antibacterial substances from larvae of the black soldier fly, *Hermetia illucens*. To induce immune responses, we septically injured the larvae with a contaminated needle. Lyophilized *H. illucens* larvae were homogenized and extracted with acidic methanol. We examined the antifungal and antibacterial effects of the low molecular weight antimicrobial factors within the larval extract on the growth of a broad range of microorganisms, including Gram-positive *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), and Gram-negative *Pseudomonas aeruginosa*. Furthermore, we isolated the anti-MRSA substances from the larval extract using high performance liquid chromatography. These investigations revealed that the larval extract possessed a broad-spectrum of antibacterial activity, demonstrating that secretions of *H. illucens* larvae prove useful in the fight against MRSA and can potentially be a source of novel antibiotic-like compounds for infection control.

**Key words:** acidic methanol, black soldier fly (*Hermetia illucens*), larval extract, MRSA, novel antibiotics.

**Introduction**

Maggot therapy is highly successful in cleansing infected and necrotic wounds (Sherman & Hall 2000) and has been practiced since the 1930s when William Baer's observations confirmed the reduction of bacterial load at the wound site by the use of maggots (Baer 1931). The main actions of fly maggots on wounds may be categorized into three modes: debridement, disinfection and bacterial death, and stimulation of tissue granulation and repair (Richardson 2004). However, the use of maggot therapy declined as antibiotics were developed and surgical techniques improved. Nevertheless, new strains of pathogenic fungi and bacteria developed primarily in response to overdose usage of antibiotics and thus were found commonly in hospitals and the wider community (Kerridge *et al.* 2005), renewing our interest in maggot therapy in the search for effective methods to treat non-healing wounds and control the evolution of resistance

(Bexfield *et al.* 2004). Recently, several studies reporting antimicrobial activity of the hemolymph and maggot extract, either in the whole body or in the excreta/secretora, have provided impetus for the development of alternative antimicrobial products into therapeutically valuable anti-infective agents (Dang *et al.* 2006). It is well known that insects have a well-developed innate immune system, subdivided into cellular and humoral defense responses, the latter of which involve production of antimicrobial peptides (AMPs) that are synthesized in the fat body and subsequently secreted into the hemolymph (Bulet & Hetru 1999; Hoffmann & Reichhart 2002).

The larvae of the black soldier fly, *Hermetia illucens*, are scavengers that can live in extremely harsh environments, such as manures and compost, inhabited by bacteria and fungi. Animal wastes and rotten plants can be decomposed and recycled by larvae. Additionally, the black soldier fly is sometimes found in carrion. These biological characteristics

suggest that the soldier fly may be rich in generations of AMPs and other substances possessing activity against drug-resistant “superbugs”. Recently, the antibacterial effect of *H. illucens* larval extract against Gram-negative bacteria was evaluated (Choi *et al.* 2012). However, insects infected with the microorganism could contain more biologically and pharmacologically active chemicals and evidence has been reported that antibacterial activity of the extract from induced larvae was much stronger than that of native larvae (Hou *et al.* 2007). The aim of the present study is to improve antimicrobial substances by inducing immunoreaction in *H. illucens* larvae and testing the antifungal and antibacterial activity of its extract against a range of Gram-positive and Gram-negative microorganisms, including the clinically important strains of methicillin-sensitive (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA), and also to isolate anti-MRSA substances from the larval extract.

## Materials and methods

### Larvae

Fifth instar larvae of the black soldier fly (*Hermetia illucens*) were obtained from Nuree Inc., Baekgok, Korea, and reared at 32°C, 62% humidity with 24 h dark cycle.

### Microorganisms

The following bacterial species were used: *Escherichia coli* (KCCM 11234), *Enterobacter aerogenes* (KCCM 12177), *Pseudomonas aeruginosa* (KCCM 11328), MRSA (methicillin-resistant *Staphylococcus aureus*), *Staphylococcus aureus* (KCCM 40881), *Bacillus subtilis* (KCCM 11316), *Kocuria rhizophila* (KCCM 11236), *Micrococcus luteus* (KCCM 11326), *Staphylococcus epidermidis* (KCCM 35494). The following fungus was used: *Candida albicans* (KCCM 11282). *Escherichia coli* and *Staphylococcus* were grown in tryptic soy broth, while other bacteria were grown in nutrient broth, and *C. albicans* in yeast malt broth.

### Preparation of the extract of the black soldier fly larvae

The larvae were dried after washing with water containing disinfectant and rinsing with sterile water. Subsequently, they were individually pricked deeply with a fine needle dipped in *S. aureus* (OD<sub>600</sub> = 2.4). The lyophilized larvae were thoroughly ground and extracted with acidified methanol (methanol/water/acetic acid; 90/9/1; v/v/v). The extract was centrifuged at 1600 × g for 10 min at 4°C and taken to dryness in a rotary evaporator under reduced pressure. Proteins and lipids were removed from the sample by sequential extraction

with chloroform and ethyl acetate. All the fractions were lyophilized and stored in a refrigerator at –20°C until use.

### Inhibition zone assay

Antimicrobial activity of Sep-Pak C18 (Waters) and high performance liquid chromatography (HPLC) eluent components were measured by inhibition zone assay as described previously (Park *et al.* 2013). Thin plates (1 mm) of 1% agarose containing  $6 \times 10^4$  cells/mL were prepared and wells of 3 mm diameter were punched out of the plates. Sep-Pak C18 and HPLC eluants were lyophilized and dissolved in distilled water at a final concentration of 1 µg/µL and 5 µL of sample was loaded into each well into appropriate wells. After overnight incubation at 37°C, the diameters of clear zones were measured.

### Minimal inhibitory concentration

Minimal inhibitory concentrations (MICs) were performed by sequential dilution in sterile 96-well cell culture plates. The microorganisms were diluted in proper medium at a starting OD<sub>600</sub> = 0.001 (approximately  $5 \times 10^5$  CFU/mL) and 90 µL dispensed to the plates. Serial dilutions of the larval extract dissolved in distilled water were loaded with 10 µL to the plate and incubated for 16 h (*M. luteus* was incubated for 42 h) at 37°C (*B. subtilis*, *E. aerogenes*, *K. rhizophila*, *M. luteus* were incubated at 30°C). The MIC was determined at 600 nm using microplate reader (EL-800, Bio-Tek Instruments, Winooski, VT, USA). All experiments were performed in quintuplicate.

### Purification of antimicrobial substances

The water-soluble extract was applied to Sep-Pak C18 and washed with distilled water (20 mL) twice and then eluted with each 20 mL of 10%, 20%, 30%, 50%, and 80% acetonitrile (ACN). The anti-MRSA fraction (10% ACN eluant) of preparative purification using Sep-Pak C18 was further purified by HPLC on a 4.6 × 250 mm Sim-pack VP-ODS (Shimadzu) connected to Futecs HPLC system with a simple linear gradient from 0.1% (v/v) trifluoroacetic acid (TFA) to 25% (v/v) ACN (0.1% (v/v) TFA) at a flow rate of 1 mL/min at room temperature. The elution pattern was monitored at 214 nm and chromatographic fractions were tested exhibiting activity against MRSA by inhibition zone assay.

## Results

### Antimicrobial activities of the larval extracts

The water-soluble extract had antibacterial activity against all the Gram-positive and Gram-negative bacteria except

*S. aureus* and *S. epidermidis*; MRSA (MIC = 25 mg/mL), *S. epidermidis* (MIC = 50 mg/mL), *K. rhizophila* (MIC = 25 mg/mL), *M. luteus* (MIC = 25 mg/mL), *B. subtilis* (MIC = 12.5 mg/mL), *E. coli* (MIC = 12.5 mg/mL), *E. aerogenes* (MIC = 25 mg/mL), *P. aeruginosa* (MIC = 12.5 mg/mL). The water-soluble extract also had antifungal activity; *C. albicans* (MIC = 25 mg/mL). The MIC against *S. aureus*, however, could not be determined even at 100 mg/mL. MIC values of the ethyl acetate extract were similar to that of the water-soluble extract, but the MIC against *S. aureus* 12256 and *E. coli* 11234 could not be determined even at 100 mg/mL. Meanwhile, the chloroform extract had no activity against all the microorganisms (Table 1). The water-soluble extract showed a broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacteria and yeast in a concentration-dependent growth manner. The water-soluble extract exhibited dramatic microbial growth at specific concentrations, but the extract exhibited concentration-dependent bacterial killing on the growth of *S. aureus* and *S. epidermidis*. Enhanced bacterial growth was observed with the water-soluble extract in low concentration (about 3 mg/mL) (Fig. 1).

### Purification of antimicrobial substances

To isolate anti-MRSA substances, the water-soluble fraction of the larval extract was applied to Sep-Pak C18 cartridges, and eluted with each 20 mL of 10%, 20%, 30%, 50%, and 80% ACN. Antibacterial activities of the Sep-Pak C18 eluants were measured by inhibition zone assay against MRSA, *E. coli*, and *B. subtilis*. Significant anti-MRSA activ-

ity was observed at the fraction eluted with 10% ACN. While the eluants did not show any antibacterial activity against *E. coli*, they exhibited a strong activity against *B. subtilis*, with the strongest inhibitory zone shown in 30% CAN. Wash1 fraction had antibacterial activity against all the tested bacteria, while wash2 fraction did not have the same activity (Fig. 2). The second step of purification was performed using HPLC with a Shim-Pack VP-ODS column (Shimadzu, Kyoto, Japan), which revealed multiple peaks, indicating the presence of various small compounds. Furthermore, large inhibitory zones against MRSA were detected from fractions 48, 61, and 78, while smaller zones were observed from fractions 50–74 including dark-brown zones (Fig. 3).

### Discussion

It is well known that the larvae of black soldier fly, *Hermetia illucens*, live in extremely harsh environments, suggesting that the soldier fly may be rich in generations of AMPs and other substances possessing activity against microorganisms, including MRSA. Previously, it has been reported that a methanol extract of *H. illucens* larvae indicated antibacterial effects against Gram-negative bacteria (Choi *et al.* 2012). However, according to a study of housefly (*Musca domestica*), antibacterial activities of the extract of inoculated larvae were two times stronger and broader than that of the native larvae (Hou *et al.* 2007). Consistent with this, our pre-experimental result also showed that the aqueous extract of *H. illucens* larvae with an immune response induced by

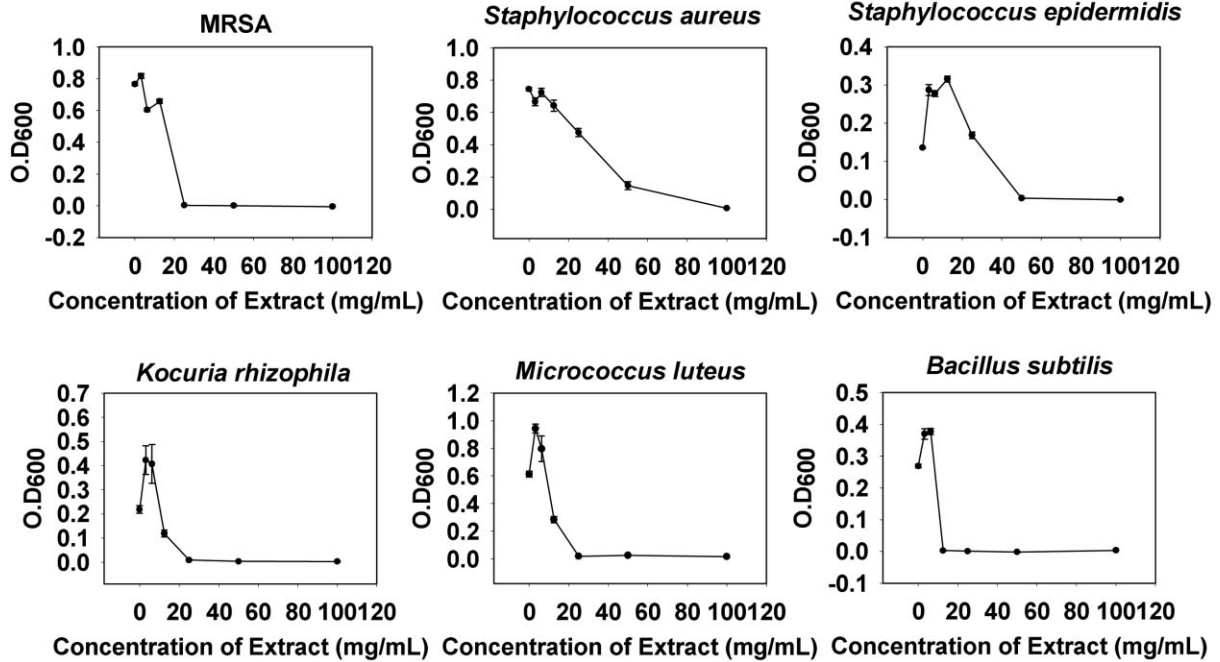
**Table 1** Minimum inhibitory concentration (MIC) of the larval extract fractions from *Hermetia illucens* larvae

Microorganisms	Strain	MIC of the extract fractions (mg/mL)			MIC of antibiotics (µg/mL)	
		Water	Ethyl acetate	Chloroform	Methicillin	Ampicillin
Gram-positive bacteria						
MRSA† (clinically isolated)		25	NT‡	>100	>80	>80
<i>Staphylococcus aureus</i>	KCCM 40881	>100	>100	>100	80	2.5
<i>S. aureus</i>	KCCM 12256	100	NT	>100	2.5	2
<i>S. epidermidis</i>	KCCM 35494	50	25	>100	>20	10
<i>Kocuria rhizophila</i>	KCCM 11236	25	NT	>100	<0.3125	<0.3125
<i>Micrococcus luteus</i>	KCCM 11326	25	NT	>100	NT	NT
<i>Bacillus subtilis</i>	KCCM 11316	12.5	25	>100	0.078125	0.078125
Gram-negative bacteria						
<i>Escherichia coli</i>	KCCM 11234	12.5	>100	>100	>80	20
<i>Enterobacter aerogenes</i>	KCCM 12177	25	NT	>100	>20	>20
<i>Pseudomonas aeruginosa</i>	KCCM 11328	12.5	25	>100	NT	NT
Yeast						
<i>Candida albicans</i>	KCCM 11282	25	50	>100	>20	>20

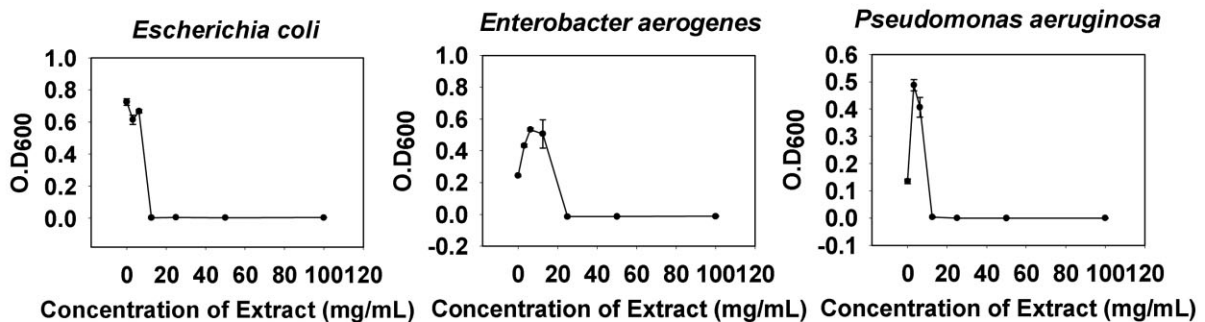
†MRSA, methicillin-resistant *Staphylococcus aureus*.

‡NT, not tested.

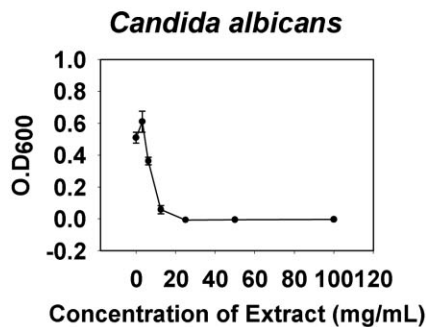
## Gram-positive bacteria



## Gram-negative bacteria

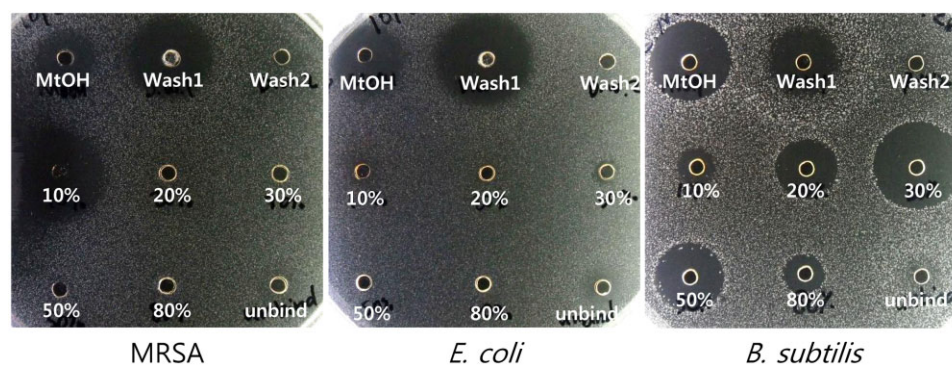


## Yeast



**Figure 1** Antimicrobial activity of water-soluble extract from *Hermetia illucens* on the growth of microorganisms at different extract concentrations. Each value is expressed as mean  $\pm$  standard error. Microorganisms were incubated in the presence of different water-soluble extract concentrations (0–100 mg/mL) for 16 h (except for *Micrococcus luteus*, which was incubated for 42 h) at 37°C (except for *Bacillus subtilis*, *Enterobacter aerogenes*, *Kocuria rhizophila*, and *M. luteus*, which were incubated at 30°C).





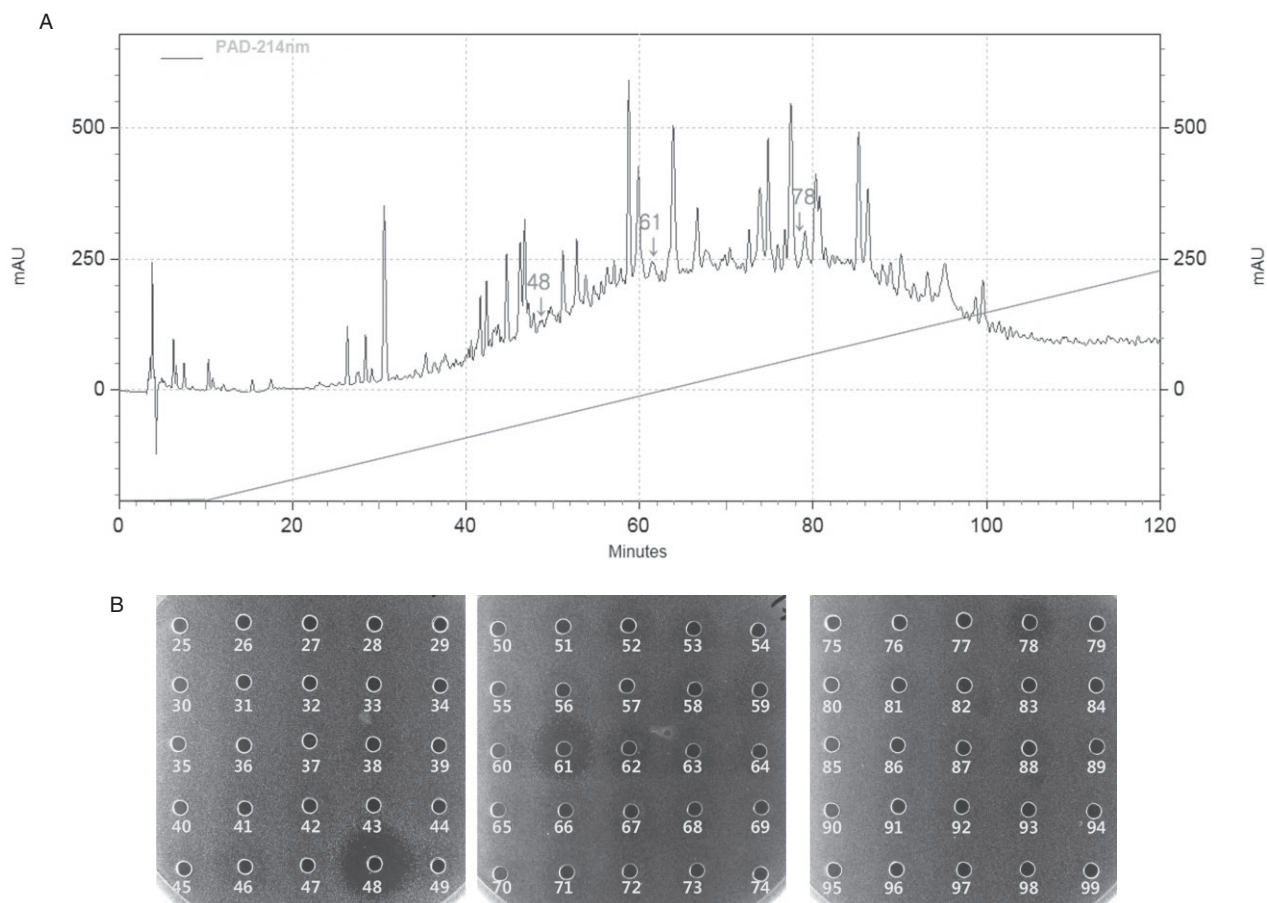
**Figure 2** Inhibition zone assay of preparative purification against methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Bacillus subtilis*. MtOH, aqueous fraction of acidic methanol extract; Wash1, 2, washing fractions of preparative purification using Sep-Pak C18; 10%–80%, 10%–80% acetonitrile (ACN) eluent; unbind, unbinding fraction.

septic needle, demonstrate stronger antibacterial activity against MRSA than that of the native larvae (data not shown).

The major goals of this study were: (i) to improve the expression of antimicrobial substances of *H. illucens* larvae inducing immune response; (ii) to extract the immunized larvae; and (iii) to test and determine their antibacterial activities. The acidic methanol extraction method was employed for the extraction of antimicrobial substances from the larvae, because the acidic methanol could denature and precipitate large proteins and polypeptides, while efficiently extracting small molecules (Meylaers *et al.* 2002). The acidic methanol extract of the larvae was sequentially extracted with chloroform and ethyl acetate to remove lipids. One of the most commonly used broth dilution techniques was employed to determine the minimal inhibitory concentration (MIC) of the extract under defined conditions. All the MIC values of the aqueous fraction of the extract were in a similar range from 12.5 to 25 mg/mL, while *Staphylococcus*, including *S. aureus* and *S. epidermidis*, demonstrated relatively lower MIC values. In particular, the MIC value of MRSA was stronger than that of *S. aureus* and *S. epidermidis*, suggesting that species-specific antibacterial substances may be expressed in *H. illucens* larvae and extracted in aqueous fraction. The chloroform fraction of the extract did not show antimicrobial activities against all tested bacteria with the result that no antimicrobial substances were extracted in this fraction. According to a previous report, the methanol extract of native *H. illucens* larvae had Gram-negative specific antibacterial activities including *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, and *Shigella sonnei* (Choi *et al.* 2012). However, the extract of acidic methanol of immunized larva used in this study demonstrated broader antimicrobial activities against all the bacteria and yeast except *S. aureus*. This appears to provide strong evidence that expression of antibacterial substances

was improved and diversified with the induced immune response. The soluble fraction of the extract was tested against 10 strains of Gram-positive and Gram-negative bacteria and a fungus in a concentration-dependent manner, revealing its broad-spectrum antimicrobial activity as shown in Figure 1. The enhanced bacterial growth observed with larval extract in concentration dependent growth assay using low concentration may also be due to the high nutritional value of the extract (Bexfield *et al.* 2004).

To isolate anti-MRSA substances, we focused on a specific fraction *H. illucens* extract, eluted at 10% ACN with Sep-Pak C18 cartridges, which showed a significant activity against MRSA. The eluants of Sep-Pak C18 did not show antibacterial activities against *E. coli*, while the soluble-extract did. This result revealed that the antibacterial substances exhibiting activity against *E. coli* passed through the column, meaning that the anti-*E. coli* substances were strongly hydrophilic in character (Fig. 2). The second step of purification was performed using HPLC with a Shim-Pack VP-ODS column, which revealed multiple peaks, indicating the presence of various small compounds. Large inhibitory zones were detected from fractions 48, 61, and 78, while smaller zones were observed from fractions 50–74 (Fig. 3). The fractions displaying strong activity must be pooled for further purification and study of structural properties of the pure substance. It should be noted that *H. illucens* larvae secrete dark-brown colored substances due to melanization, or biosynthesis of melanin, a phenolic biopolymer involved in the insect immunity. The cytotoxic phenols have already been investigated extensively (Sugumaran 2002) and are well established as compounds displaying broad-spectrum antibacterial effect and, therefore, the presence of these antibacterial compounds in aqueous extract of *H. illucens* is possible (fractions 50–74). Our results show that the water-soluble fraction of the whole-body extract possessed



**Figure 3** High performance liquid chromatography (HPLC) purification of the 10% acetonitrile (ACN) solid phase fraction, as prepared from aqueous extract of *Hermetia illucens*. (A) Chromatogram of HPLC purification using Sim-pack VP-ODS. The fractions exhibiting activity potent against methicillin resistant *Staphylococcus aureus* (MRSA) were represented by arrows. (B) Inhibition zone assay analysis of the fractions against MRSA.

significantly stronger antibacterial activity against MRSA, suggesting the presence of more than one “antibacterial” substance acting in synergy to increase the effect. Additionally, the aqueous extract of *H. illucens* has proven to be highly robust, capable of withstanding several freeze–thaw cycles and lyophilization, and is stable as a freeze-dried preparation, all of which are important properties in terms of development of a product for pharmaceutical purposes. It has been recently reported that the presence and production of a new class of small, perhaps semi-peptidergic antimicrobial substances may not be restricted to the hemolymph or fat body and therefore, when attempting to isolate those compounds, preparation of whole-body extract could be more efficient (Meylaers *et al.* 2002). A number of such small, low molecular weight antimicrobial dipeptides have been purified from Dipteran species. For example, the inducible antibacterial compound N-b-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD,

573 Da) was isolated from the adult fleshfly, *Sarcophaga peregrine* (Leem *et al.* 1996), and two small substances,  $\beta$ -alanyl-tyrosine (252 Da) and 3-hydroxykynurenine (224 Da), were isolated from larvae of the grey fleshfly, *Neobellieria bullata* (Meylaers *et al.* 2003). Other low molecular weight antimicrobial compounds reported from insects include *p*-hydroxycinnamaldehyde (148 Da), isolated from induced larvae of the sawfly, *Acantholyda parki* (Leem *et al.* 1999), and 1-lysophosphatidylethanolamine (451.2 Da) from native larvae of the housefly, *Musca domestica* (Meylaers *et al.* 2004).

In conclusion, all these features provide important evidence that larval secretions of *H. illucens* are a very rich source of substances with novel antimicrobial properties that could be highly useful in the fight against MRSA and control of other nosocomial infections. Further research on the characterization and purification of the extract may contribute considerably to the development of new antibiotics.

## Acknowledgments

The present research was conducted by the research fund of Dankook University in 2012.

## References

- Baer WS (1931) The treatment of chronic osteomyelitis with the maggot (larva of the blow fly). *Journal of Bone and Joint Surgery* **13**: 438–475.
- Bexfield A, Nigam Y, Thomas S, Ratcliffe NA (2004) Detection and partial characterisation of two antibacterial factors from the excretions/secretions of the medicinal maggot *Lucilia sericata* and their activity against methicillin-resistant *Staphylococcus aureus* (MRSA). *Microbes and Infection* **6**: 1297–1304.
- Bulet P, Hetru C (1999) Antimicrobial peptides in insects: structure and function. *Developmental and Comparative Immunology* **23**: 329–344.
- Choi W-H, Yun J-H, Chu J-P, Chu K-B (2012) Antibacterial effect of extracts of *Hermetia illucens* (Diptera: Stratiomyidae) larvae against Gram-negative bacteria. *Entomological Research* **42**: 219–226.
- Dang X-L, Tian J-H, Yi H-Y *et al.* (2006) Inducing and isolation of antimicrobial peptides from oriental fruit fly, *Bactrocera dorsalis* Hendel. *Insect Science* **13**: 257–262.
- Hoffmann JA, Reichhart JM (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology* **3**: 121–126.
- Hou L, Shi Y, Zhai P, Le G (2007) Antibacterial activity and in vitro anti-tumor activity of the extract of the larvae of the housefly (*Musca domestica*). *Journal of Ethnopharmacology* **111**: 227–231.
- Kerridge A, Lappin-Scott H, Stevens JR (2005) Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*. *Medical and Veterinary Entomology* **19**: 333–337.
- Leem JY, Jeong IJ, Park KT, Park HY (1999) Isolation of *p*-hydroxycinnamaldehyde as an antibacterial substance from the saw fly, *Acantholyda parki* S. *FEBS Letters* **442**: 53–56.
- Leem JY, Nishimura C, Kurata S, Shimada I, Kbayashi A, Natori S (1996) Purification and characterisation of N-b-alanyl-5-Sglutathionyl-3-4-dihydroxyphenylalanine, a novel antibacterial substance of *Sarcophaga peregrina* (fleshfly). *Journal of Biological Chemistry* **271**: 13573–13577.
- Meylaers K, Cerstiaens A, Vierstraete E *et al.* (2002) Antimicrobial compounds of low molecular mass are constitutively present in insects: characterisation of  $\beta$ -alanyl-tyrosine. *Current Pharmaceutical Design* **8**: 99–110.
- Meylaers K, Cerstiaens A, Vierstraete E *et al.* (2003) Antimicrobial compounds of low molecular mass are constitutively present in insects: characterization of b-alanyl-tyrosine. *Current Pharmaceutical Design* **9**: 159–174.
- Meylaers K, Clynen E, Daloz D, DeLoof A, Schoofs L (2004) Identification of 1-lysophosphatidyl-ethanolamine (C<sub>16:1</sub>) as an antimicrobial compound in the housefly, *Musca domestica*. *Insect Biochemistry and Molecular Biology* **34**: 43–49.
- Park S-I, An HS, Chang BS, Yoe SM (2013) Expression, cDNA cloning, and characterization of the antibacterial peptide cecropin D from *Agrius convolvuli*. *Animal Cells and Systems* **17**: 23–30.
- Richardson M (2004) The benefits of larval therapy in wound care. *Nursing Standard* **19**: 70, 72, 74.
- Sherman RA, Hall MJR (2000) Medicinal maggots: an ancient remedy for some contemporary afflictions. *Annual Review of Entomology* **45**: 55–81.
- Sugumaran M (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigment Cell Research* **15**: 2–9.