Antibacterial Activity of Excretions-Secretions from *Chrysomya megacephala* Against *Escherichia coli*

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Background: *The blowfly, Chrysomya megacephala, is distributed worldwide. Previous studies found maggot excretions-secretions from other blowfly species inhibited pro-inflammatory response and antimicrobial activity.*
Objective: *This study aimed to test the bactericidal activity of excretions-secretions from C. megacephala larvae.*
Material and Method: *A total of 1,500 3-day-old larvae were used to collect excretions-secretions (ES) modified by the Barnes method. The bactericidal activity of the excretions-secretions was test by Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli using suitable liquid culture assay. Scanning electron microscope (SEM) was used to investigate the morphological change of the bacteria.*
Results: *E. coli were significantly inhibited by excretions-secretions from C. megacephala larvae. P. aeruginosa and S. aureus were not found to inhibit growth.*
Conclusion: *The excretions-secretions from C. megacephala larvae may have a medical property for the inhibition of bacterial growth.*

Keywords: *Chrysomya megacephala, Excretions-secretions, Antimicrobial*

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Maggot therapy is an ancient bio-therapeutic method used in the healing of chronically-infected wounds. It has been successfully used to treat non-healing wounds which have previously failed to respond to conventional treatment[1]. This treatment method employed the use of freshly emerged, sterile larvae of the common green bottle fly, *Lucilia sericata*[2-4]. Currently, the limitations on production of sterile maggots and a lack of knowledge about their benefits make the cost of treatment per patient in Thailand expensive. It was found that excretions-secretions (ES) derived from larvae of blowfly and other fly species had antimicrobial activity against several pathogenic bacteria[5,6]. However, research on the fly and bacterial interactions native to Thailand have not been reported.

The objective of this study was to study the excretions-secretions from third instar larvae of the blowflies *Chrysomya megacephala*, the dominant synanthropic flies collected in Thailand, on anti-microbial action in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*.

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Material and Method

Production of larval excretory-secretory

ES from laboratory strains of *C. megacephala* larvae were modified from Barnes et al (2010)[7]. Eggs laid on fresh pork were washed with 70% alcohol followed by sterile distilled water three times successively. The treated eggs were deposited on fresh pork and allowed to hatch to larvae within 24 hours. Early third (3 day-old) instar larvae were aseptically transferred to a flat petri-plate followed by washing with 70% alcohol and triplicate sterile distilled water washes and soaked on filter paper (Whatman No. 1). The treated larvae were transferred to a 15 ml sterile tube with de-ionized water to a density of 100 larvae per 200 µl of de-ionized water. They were allowed to incubate in the test tube in the dark at 30°C for 1 hour. Subsequently, the resultant larval extracted ES products were filter-sterilized through 0.2 µm filter. The sterility was verified by the preparation of an aliquot for

microbial viable counts using Muller Hinton agar plates as the culture media.

Bacterial strains

P. aeruginosa ATCC27853, *S. aureus* ATCC25923, and *E. coli* ATCC25922 were cultured in nutrient agar (Criterion, Santa Maria, USA, Hardy Diagnostics) and were then incubated at 37°C for 24 hours. The single colonies were further transferred into 3 ml of trypticase soy broth (Criterion, Santa Maria, USA, Hardy Diagnostics) and incubated at 37°C for 3 hours. The bacterial suspension was adjusted to McFarland No. 0.5 (media containing bacteria at $1x10^8$ CFU/ml). The bacterial suspension was further diluted 10-fold serially to 10^5 CFU/ml. A hundred µL of bacterial suspension was applied onto Muller Hinton Agar (Criterion, Santa Maria, USA, Hardy Diagnostics) for plate counting.

Antimicrobial activity test of the extract

Bactericidal activity

The amounts of 100 µL and 300 µL of the extraction were added to 3 ml of bacterial suspension. Controls were used consisting of 100 and 300 µL of de-ionized water. The test and control were incubated at 37°C for 18 hours. After this time, 100 µL of bacterial suspension was applied onto Muller Hinton agar for plate counting. Bacterial cell counts were determined by counting the colonies after the plates were incubated at 37°C for 24 hours.

Optical density assay

The bacterial suspension was measured by optical density at 450 nm (OD_{450}) at hourly intervals from time zero to 18 hrs after incubation.

Scanning electron microscope investigation of the morphological change of bacteria

The samples from the test of *P. aeruginosa*, *S. aureus*, and *E. coli* (300 µL of de-ionized water) and their control were dropped on 0.45 µm filter membrane and dried at room temperature. All samples were fixed in 2.5% glutaraldehyde in phosphate buffer (PBS) at pH 7.2 at 4°C for 24 hours. After primary fixation, samples were rinsed two times with PBS at 15 minute intervals. The rinsed samples were then placed in 1% osmium tetroxide as a post-fixative at room temperature for 1 hour. Subsequently, they were rinsed twice with PBS and dehydrated gradually at 30 minute intervals with increasing concentrations of alcohol of 10%, 30%, 50%, 70%, 80%, 90%, 95%, and twice with 100% to replace water with ethanol. Critical point drying (CPD) was performed. Specimens were then attached to aluminum stubs with double-stick tape and coated with gold (Au) in a sputter-coating apparatus before being viewed with an SEM (JEOL: Japan) at the College of Medicine and Public Health, Ubon Ratchathani University.

Statistical analysis

Statistical analysis was performed using Chi-square test to compare the inhibition for cells and Fisher's exact test for optical density in each group. Data were considered statistically significant when $p<0.05$.

Results

Bactericidal activity

Three bacterial strains were tested by bactericidal activity. The ES from *C. megacephala* larvae showed a significantly reduced *E. coli* ($p<0.001$) count after 18 hours incubation when compared to the control test (Table 1). The 300 µL of ES was more effective to reduce the bacterial count than 100 µL (Table 1 and 2). The maggot ES was not found to inhibit *P. aeruginosa*, and *S. aureus* growth. Moreover, *S. aureus* had a significantly ($p<0.001$) increased viable cell count after 18 hours incubation with ES (Table 1).

SEM investigation confirmed ES from *C. megacephala* inhibited *E. coli* growth (Fig. 1). The

Table 1. Antibacterial activity of excretions-secretions from *C. megacephala* larvae to *P. aeruginosa*, *S. aureus*, and *E. coli*

Excretory-secretions	*P. aeruginosa* ATCC 27853 (CFU/ml)		*S. aureus* ATCC 25923 (CFU/ml)		*E. coli* ATCC 25922 (CFU/ml)	
	At 0 hour	At 18 hours	At 0 hour	At 18 hours	At 0 hour	At 18 hours
100 µL	$4.30x10^2$	$3.10x10^3$	$1.46x10^3$	$4.20x10^3$	$4.25x10^3$	$3.20x10^3$
300 µL	$4.30x10^2$	$3.10x10^3$	$1.46x10^3$	$3.50x10^3$	$4.25x10^3$	$2.20x10^3$

Table 2. Optical density at 450 nm of bacteria cultured with excretions-secretions from *C. megacephala* larvae

Organism	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
Control at 18 hours	0.701	0.118	1.677
Test 300 µL at 18 hours	0.846	0.837	0.835

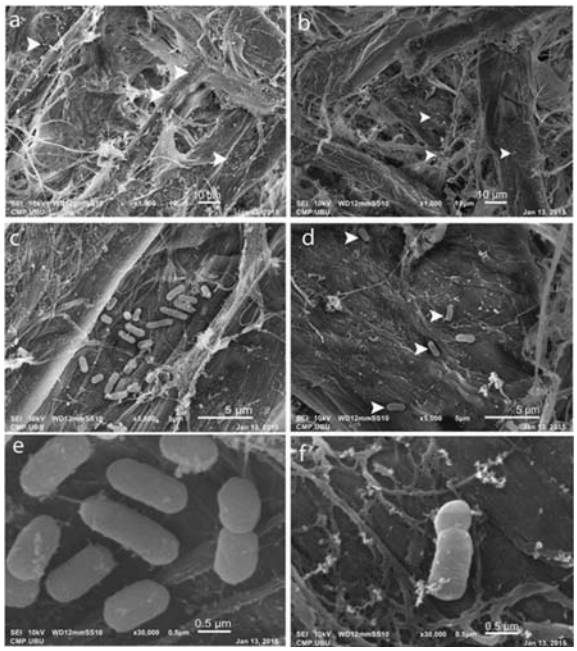

Fig. 1 SEM micrograph of *E.coli* treated with excretions-secretions from *C. megacephala* larvae of *C. megacephala*, (A,C) *E. coli* control revealed numerous bacteria (arrow head), (B,D) *E. coli* treated with ES presented a few bacteria (arrow head), (E) higher magnification of *E. coli* control, and (F) higher magnification of *E. coli* treated with ES showing a smooth surface.

SEM micrograph of the treated *E. coli* appeared to have a lower number of bacteria than the untreated or control (Fig. 1A-D). However, higher magnifications of *E. coli* of both treated and control cases were not different and revealed a smooth surface of bacteria (Fig. 1E-F).

Discussion

The study showed that ES of *C. megacephala* larvae had potent antimicrobial activity against *E. coli* by viable count and SEM. Optical density was not significantly different ($p = 0.6580$) between the groups. Inhibition of growth was not observed in *P. aeruginosa* and *S. aureus*. Moreover, *P. aeruginosa* and *S. aureus* had significantly increased viable cell counts when treated with ES for 18 hours. The findings of the study contrasted with ES of *Lucilia sericata*, this excretion having potent antibacterial activity to multidrug resistance. Methicllin resistant *staphylococcus aureus* (MRSA) treated with ES of *L. sericata* showed significant inhibition of bacterial growth[6]. This study tested the non multi-drug resistant bacteria, *S. aureus* ATTCC25923, treated with ES of *C. megacephala* and showed greater bacteria count and turbidity after treatment for 18 hours. Moreover, ES of *L. sericata* inhibited *P. aeruginosa*, *E. coli*, *Bacillus cereus*, and *Proteus mirabilis* growth[7]. Differences in the strains of maggots may be involved in differences in antibacterial activity.

Although ES of *C. megacephala* did not show a broad spectrum of antibacterial activity against both gram-positive and negative bacteria, the secretion significantly inhibited only the *E. coli* growth. These three bacterial strains were different in structure and morphology. *S. aureus* was gram-positive cocci in cluster bacteria and peptigoglycan was a major cell wall component. In part, *E. coli* and *P. aeruginosa* were gram-negative bacilli and peptigoglycan and lipopolysaccharide were cell wall components. *E. coli* fermented lactose but *P. aeruginosa* did not ferment these sugars. The different characteristics of these bacteria contributed to the action of ES. ES of *C. megacephala* was able to act against *E. coli* by doses of ES both 100 and 300 µL respectively. The 300 µL of ES showed significantly more inhibition of bacterial growth than 100 µL. SEM examination confirmed ES from *C. megacephala* inhibited *E. coli* growth. Nevertheless, the ES from this study did not destroy the surface of the bacteria as observed by using SEM. However, the researchers did not know the exact bioactive compounds in the ES. Further study plans to collect larger amounts of larvae to prepare the ES and stock by lyophilize it, then test the toxicity of the cells, and study the chemical structures.

ES of *C. megacephala* larvae showed activity

against *E. coli*. However, other members of enterobacteriaceae need to be further investigated.

Conclusion

This study screened the antimicrobial activity of ES from *C. megacephala* larvae. The ES treatment inhibited only *E. coli* growth. *P. aeruginosa* and *S. aureus* did not experience growth inhibition. Currently, ES from *C. megacephala* larvae controls the growth of *E. coli* and is a new chemical target for future control of *E. coli* infection.

What is already known on this topic?

Maggot therapy has been shown to be a safe and effective means of chronic wound management in many countries of the world. Much of the application of the therapy focuses on the use of *Lucilia sericata*, a fly species not found in Thailand. It has been found that ES derived from larvae of blowfly and other fly species have antimicrobial activity against several pathogenic bacteria, however, research on the fly and bacterial interactions native to Thailand has not been reported.

What this study adds?

We investigated the antibacterial activity of the ES of the blowflies *C. megacephala*, the predominant synanthropic flies collected in many parts of Thailand. The result showed that the ES from the larvae of *C. megacephala* inhibited *E. coli* growth.

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Potential conflicts of interest

None.

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ฤทธิ์ต้านแบคทีเรียของสารคัดหลั่งจาก *Chrysomya megacephala* ต่อเชื้อ *Escherichia coli*

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ภูมิหลัง: แมลงวันหัวเขียวชนิด *Chrysomya megacephala* พบแพร่กระจายอยู่ทั่วโลก การศึกษาก่อนหน้านี้พบว่าสารคัดหลั่งจากแมลงวันหัวเขียวสามารถยับยั้งการตอบสนองอักเสบและมีฤทธิ์ต้านเชื้อแบคทีเรีย

วัตถุประสงค์: ทดสอบสารคัดหลั่งจากแมลงวันหัวเขียวชนิด *C. megacephala* ในการต้านเชื้อแบคทีเรีย

วัสดุและวิธีการ: นำตัวอ่อนของแมลงวันหัวเขียวที่มีอายุ 3 วันทั้งหมด 1,500 ตัวมาสกัดสารคัดหลั่งโดยการดัดแปลงมาจากวิธีของ Barnes ทดสอบฤทธิ์ต้านแบคทีเรียสามสายพันธุ์ได้แก่ *Pseudomonas aeruginosa, Staphylococcus aureus,* และ *Escherichia coli* โดยการเลี้ยงในอาหารเหลวที่มีส่วนผสมของสารสกัดปริมาณ 100 mL และ 300 mL แล้วนำไปนับจำนวนเชื้อที่มีชีวิตบน Muller hinton agar plate พร้อมทั้งตรวจดูการเปลี่ยนแปลงรูปร่างของเชื้อแบคทีเรียด้วยกล้อง Scanning electron microscopy (SEM)

ผลการศึกษา: *E. coli* ถูกยับยั้งด้วยสารคัดหลั่งจากตัวอ่อนของแมลงวัน *C. megacephala* เชื้อ *P. aeruginosa,* และ *S. aureus* ไม่พบการยับยั้งการเจริญเติบโต

สรุป: สารคัดหลั่งจากตัวอ่อนของแมลงวัน *C. megacephala* อาจจะมีคุณสมบัติทางการแพทย์ในการยับยั้งการเจริญเติบโตของแบคทีเรีย