Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2021; 9(6): 14-19
© 2021 JEZS
Received: 18-08-2021
Accepted: 26-09-2021

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Antibacterial activity of secretion/excretion blow fly, *Callifora vomitoria* (Diptera: Calliphoridae) third instar larvae *in vitro*

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Abstract
The antibacterial activity of excretion/secretion (ES) from flesh feeding blow fly, *Calliphora vomitaria* L. were relatively compared with that of Ceftriaxone (CRO) as standard drug. The larval ES was obtained from both normal fed larvae and others cannibalized infected larvae with the four studied pathogenic bacteria. Diffusion disc test was used to assess ES viability against the sensitive and piperacillin resistant bacterial species; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa* and *Klebsiella pnuemuoniae*. The antibacterial potency of the two fractionation phases of ES in DMSO were varied among that four species. This potency depends upon the fractionated ES phase. Generally, dense (upper) and clear (lower) phases of normal ES more effective against Gram-positive than Gram-negative species, while not that for phases of ES collected from cannibalized infected larvae. It was found the growth inhibition zones significantly very varied between antibacterial activity of ES and CRO. Overall, the present result gives opportunity promise for qualitative evolution new resources against antimicrobe from larvae products.

Keywords: *Calliphora vomitaria*, excretion, secretion, antibacterial, resistant bacteria

Introduction
Carrions and corpus are suitable sources for nutrition and oviposition sites to some decomposing insect species, In addition innate immunity system, which represented by humoral and cellular immunity and that including melanization, phagocytosis, nodulation and encapsulation [14] and [24], some insects were supplied with external defense equipment characterized by antimicrobial activity, for instances oral and anal secretions of carrion beetles inhibited growth Gram-positive and Gram-negative bacteria [11] and [9]. Many dipteran larvae are exposed to stress of pathogenic microorganisms, so, larval excretions/secretions giving optimal activity against wide-spectrum of bacteria [12] and [7]. *Lucilia* and *Calliphora* are important genera in calliphorid family, and they depending in forensic entomology [4] and [5], these larvae have selective adaptation in contaminated environment with microbes, so, protected themselves by antimicrobial peptides (AMPs) which done separately and in synergistic each other [21] and [6], or potentiation by enhancing antimicrobial activity of others [28]. Now these AMPs are be considered strong natural antibacterial inhibitors [29]. Also, the extracted lipid compounds from *Calliphora vicina* and *Calliphora vomitoria* had antimicrobial activity [8]. Excretion/secretion (SE) of *Lucilia sericata* larvae are use in healing necrotic wounds. ES were prevented biofilm formation and degraded biofilms initiation by the bacteria *Staphylococcus aureus* and *Pseudomonus aeruginosa* [27].
First attention was paid to the beneficial effect of larval ES early in 1930 [23] was found that after 5 to 10 min – exposure, significant antimicrobial activity of non - sterile larvae of *L. sericata* against some pyogenic bacteria. The insects have ability to resist microbial infection [15]. Injured *L. sericata* larvae were stimulated to produce lucifencin AMP with antibacterial activity [26]. AMP are mainly produced by fat bodies and hemocytes and released to hemolymph [13] and [30]. AMPs are antibacterial molecules and important ingredients in the ES of the larval stage, besides to digestive enzymes which used in external digestion [19]. The antibacterial molecules have been identified whether their sources SE or body extract [5].
Since the scientific research signals application the hypothesis, carrion insects are protect themselves against microbial infection by antimicrobial molecules. In this scope, the present study aims to test the antibacterial activity SE of late instar larvae *Calliphore vomitoria* on

growth inhibition the pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa* and *Klebsiella pneumonia in vitro*.

Materials and Methods

Materials

Insect colony

Out door and nearby Entomology laboratory, the colony of the blue bottle fly, *Calliphora vomitoria* was established in semi-field condition. The cage dimensions were 2×1×1 m with wooden floor and sheltered for the colony maintenance, wild adult flies by net were collected from city slaughter in November 2020 and fed on 15% honey bee solution, fish remaining filth and ovaries were presented from local fishing market and used for larval feeding and ovipositing sites. Wet sandy soil was placed in cage beside feeding site for pupation the wandering full grown larvae.

Bacteria

The bacteria were used as marker to evaluate the antimicrobial activity of larval excretion/secretion. The pathogenic bacteria; *Staphylococcus aureus* (Gram-positive), *Escherichia coli*, *Pseudomonus aeruginosa* and *Klebsiella pneumonia* (Gram-negatives) were obtained as a loan from Dr. Alaa Taha Younis, Microbiology lab / Research unit / Biology Department / Collage of Education for Pure Science / Mosul University /Iraq.

Culture media

From NEOGEN Culture Media, Muller-Hinton media were purchased and culture prepared for bacteria inoculation

Methods

Preparation of excretion / secretion

The excretion/secretion (ES) were collected according to [3] and [18] with modifications. Approximately 1000 larvae (700 gm) in the late third stage were picked out from caged colony (plate 1). The larvae were preserved in enamel dish (25 × 25×20 cm), covered and tied with mesh clothe and left for overnight under the laboratory conditions. The larvae were rinsed by 100 ml of the negative solvent DMSO with 7: 1 (w\v) ratio. After removing the larvae, the obtaining crude were filtrated with wattmann filter paper No. 1 under low pressure. The filtrate was centrifuged at 3000 rpm for 15 min., and fractionated into two phases, they can be physically distinguished as upper deep and lower pale pink color (plate 1E). With about ration 20: 80 between the two rested phases.

Plate 1: Experimental martials of *Calliphora vomitoria* for preparing the excretion and secretion (SE); A – Ova, B – feeding larvae, C – collected larvae, D – larval cannibalism on infected larvae and E – ES filtrated phases.

Excretion and secretion of infected larvae

Infected *C. vomitoria* larvae were obtained by feeding them on another dead larvae as fellow; 150 larvae at second instar were took from the colony and fed on broth agar media contaminated with mixture of the four studied marker bacteria; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa* and *Klebsiella pneumoniae*. Later, the grown third instar larvae were killed by cooling, and introduced as food to the developing experimental 150 second instar larvae plate 1C). The feeding larvae on dead others were grew and became in third instar. Then, same protocol to prepare excretion / secretion of uninfected larvae has been followed.

Antibacterial test

Bacteria growth inhibition by third larvae ES were *in vitro* tested on the marker bacteria, by agar diffusion discs, the test was modified after Kirby-Bauer [2], to estimate bacteria susceptibility. From each marker bacteria plate, samples were swapped on the petri dish plates, on each plate positive and negative controls and treated discs were fixed with three replicates, the discs were previously treated with three concentrations of ES were 0.05, 0.1 and mg/ml of each the two phases of the ES stock solution, and dissolved in 1.0 ml of the inert DMSO solvent. The plates were incubated for 24 hrs. at 37°C, the diameter of growth inhibition zones were measured by caliper in mm, and exclusive 5 mm (diameter of

the disc) of the readings. For evaluating the antibacterial activity of the ES, the growth inhibition zones were ranked after [26]. into the following grades: ≥ 8 mm good, 6-8 mm moderate, 4-5 mm and 2-3 mm very week.

Results

Antibacterial activity of excretion / secretion (ES) of blue-bottle *Callifora vomitoria* were studied against sensitive and resistant Gram-Positive *Staphylococcus aureus* and Gram – negatives; *Escherichia coli, Pseudomonus aeruginosa* and *Klebsiella pneumonia*e. The two main sources of the investigated ES were obtained from uninfected larvae and others infected with same experimental bacteria. Table 1 illustrates that the inhibition zone for sensitive bacteria resulting by the two supernatant phases of ES normal fed larvae in comparison with CRO antibiotic as positive control. The clear (lower) phase have significant growth inhibition activity among the treated bacteria, the diameters of the inhibited zones were 21.5, 5.5 17.2 and 13.3 mm for the Gram - positive bacteria *S. aureus*, and Gram – negatives *E. coli, P. aeruginosa* and *K. Pneumoniae* respectively. Antibacterial activity of the dense (upper) phase less than that of the clear phase, and these inhibition zones with the same bacterial sequence were: 17.2, 12.8, 11.8 and 9.9 mm. On the other hand, ES of the cannibalized infected larvae were showed in Table 2, their inhibition zones 28.3, 21.8, 336.2 and 17.3 mm at lower SE phase, while inhibitions zones of the upper phase were 25.0, 17.2, 23.2 and 12.8 mm for the bacteria *S. aureus, E. coli, P. aeruginosa and K. Pneumoniae* respectively. Inhibition growth zones by CRO antibiotic were less then zone diameter of the two phase of infected or normal fed larvae (9.2, 3.3, 6.8 and 3.5 mm) for *S. aureus, E. coli, P. aeruginosa and K. Pneumoniae* bacteria.

Plate 2: Growth inhibition zones of *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli* were treated by clear (C) and dense (D) SE fractions of *Calliphora vomitoria* larvae. CRO – Ceftriaxone.

Antibacterial activity against resistant bacteria

Inhibition growth zones of the resistant bacteria *S. aureus, E. coli, P. aeruginosa and K. Pneumoniae* to piperacillin antibiotic were measured after treatment with two concentrations of each dense and clear phases of *C. vomitoria* ES. Table 3 shows the bacterial inhibition at the concentrations 0.1 and 0.05 mg/ml at clear (lower) phase for the resistant bacteria *S. aureus, E. coli, P. aeruginosa and K. Pneumoniae* were (13.8, 10.0 mm), (9.4, 5.5 mm), (16.8, 10.7 mm) and (14.0, 3.5mm) respectively at the concentrations. Besides, the clear (lower) phase of the ES were contenued in antibacterial action but not proportionally with Concentration multiplication, as in dense phase application, so the inhibition growth zones; (15.2, 9.2 mm), (11.4, 9.9 mm), (12.8, 9.8mm) and (9.5, 4.2 mm) of the resistant bacteria (table 2). The +ve control (CRO antibiotic) had negligible effect (plate 3) on the treated resistant bacteria *S. aureus,E. coli, P. aeruginosa and K. Pneumoniae*.

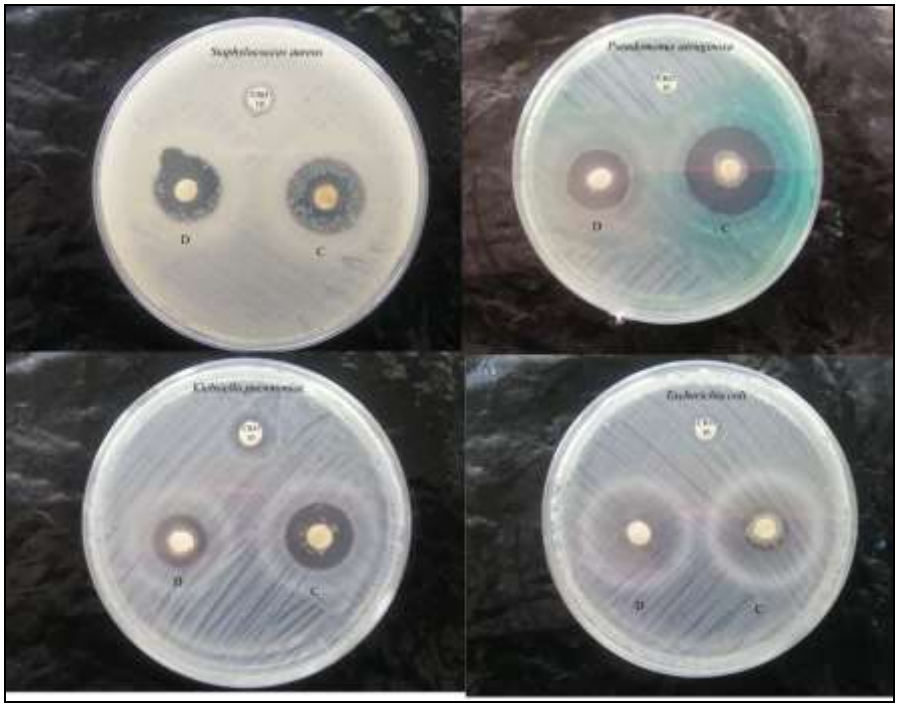

Plate 3: Antibacterial activity of two SE fractionation (clear and dense) phases of *Calliphora vomitoria* on Piperacllin resistant bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*. C – clear, D – dense phases. CRO – Ceftriaxone

Table 1: Antibacterial activity of 0.05 mg\ml ES of *Clliphore vomitoria* third larvae

Secretion/Excretion of uninfected bacteria	Growth zone (mm) of Inhibition			
	Gram- positive	Gram- Negatives		
	S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
Lower phase	18.5±0.5 a	12.2±0.8 bc	11.2± 1.3 b	10.3±1.5 c
Upper phase	17.2±1.2 a	12.8±0.3 c	10.8±0.3 cd	9.9±0.8 d
CRO (+ve)	9.2±0.8 a.	.3±1.0 c*3*	6.3±1.0 c	.3±1.0 c*4*

- Horizontal means ± SD with different letters are significantly different of P ≤ 0.05 (Duncan, s test). CRO is Ceftriaxone antibiotic (standard drug).

Table 2: Growth inhibition of marker bacteria by antibacterial of 0.05 mg\ml ES of *Clliphore vomitoria* third larvae cannibalized bacteria infected larvae

E/S of infected larvae	**Inhibition growth zone (mm) of**			
	Gram- positive	**Gram - Negatives**		
	S. aureus	***E. coli***	***P. aeruginosa***	***K. pneumoniae***
Lower phase	28.3±0.6 b	21.8±1.0 c	33.2±1.0 a	17.3±1.5 c
Upper phase	25.0±0.5 a	17.2±0.8 b	23.2±0.8 ab	12.8±0.3 c
CRO (+ve)	9.2±0.8 a	3.3±1.0 c	.8±0.3 *6*	3.5±0.5 c

-Means in Horizontal view with different letters are *significantly* different letters are significantly different of P ≤ 0.05 (Duncan, s test) -CRO is Ceftriaxone antibiotic (standard drug).

Table 3: Growth inhibition of marker bacteria by antibacterial of piperacillin resistant with ES of *Clliphore vomitoria* third larvae instar larvae at 0.1and 0.05 mg\ml concentrations.

SE/Phase Conc. (mg/ml)		**Inhibition growth zone (mm) of**			
		Gram- positive	**Gram- Negatives**		
		S. aureus	***E. coli***	***P.aeruginosa***	***K. pneumoniae***
Clear phase	0.1	13.8±0.8 b	9.4±0.1c	16.8±0.5 a	14.0±0.8 ab
	0.05	10.0±0.5 a	5.5±1.5 bc	10.7±0.3 a	4.5±1.0 c
Dense phase	0.1	15.2±0.3 a	11.4±0.5 c	12.8±1.0 b	9.5±0.5 d
	0.05	9.2±0.8 a	6.9±0.5 b	8.8±0.8 a	4.2±1.5 c
CRO (+ve)		0.0±0. 0	0.0±0. 0	0.0±0. 0	0.0±0. 0

- Means in horizontal view with different letters are significantly different at P ≤ 0.05

Discussion

The results of this study are consistency with the hypothesis that the carrion insects were adapted with microbial stress and their immune system respond through anal excretions, oral excretions and internal secretions for inhibiting pathogenic agents: bacteria, fungi and yeasts. *Calliphora vomitoria* larvae are obligate feeder on carrions. Our finding were supported that hypothesis within excretion/ secretion elution of the 3rd instar larvae by inert solvent (DMSO) and tested them against known sensitive and Piperacillin resistant bacteria. Positive control CRO antibiotic – standard drug - usage to evaluate antimicrobial action of the crude ES to build millstone for

next generation of alternative antibacterial molecules against today antibiotic resistant bacteria.

After ES fractionation, the two phases (dense and clear) were varied in an antibacterial activity against the Gram-positive *S. aureus* and Gram-negatives; *E. coli, P. aeruginosa* and *K. pneumoniae.* Table 1 shows that growth inhibition zones are in good effect according to [26] taxa, the clear phase was significantly more effective than dense phase, and more inhibited Gram-positive bacteria (*S. aureus*), than Gram-negatives (*E. coli, P.aeruginosa* and *K. Pneumoniae*). Clear and dense phases are more effective than the positive control (CRO) with valuable significance. In otherwise, inhibition zones of *P. aeruginosa* were 33.2 and 20.2 mm and for *K. Puenmoniaein*.20.3 and 15.8 mm at clear and dense phases treatments, while those bacterial were not affected by CRO treatment. However dense and clear ES phases of stressed larvae which cannibalized infected larvae appeared high significant growth inhibitions (zone diameters), looking for the table 1 and 2 be saw the differences in growth inhibition zone values between the antimicrobial molecules so were from stressed (infected) or those without stress (uninfected) *C. vomitoria* larvae. The present finding were supported by [10] and [1] were SE *L. sericata* and *C. vicina* inhibited growth of *S. aureus, P. aeruginosa* and *K. Pnuemoniae* and other microorganisms.

Today, resistant bacteria strains are demand inspecting for alternatives to solve problem of drug resistant. Therefore, figure 1 exhibits that ES of third larvae of *C. vomitoria* in either two applied phases (dense, clear) had meaningful efficacy against piperacillin resistant Gram positive and Gram negative marker bacterial strains in comparison with no effecting by CRO antibiotic. Our results were contest with [19], they found that induced immune activity of black soldier fly, *Hermtie illucens* larvae with contaminated needle, and isolated compounds from larvae extract caused antibacterial activity on sensitive and meticillin *S. aureus* and *P. aeruginose*. Otherwise, [17] were reported that insect body extracts have antibacterial effect on Multi drug Resistant bacteria. The present study was agreed with other articles were two AMPs identified from *Pichia pastoris* with high antimicrobial potency against Methicillin resistant *S. aureus* and other Gram positive bacteria [16], and feeding carrion and corpus maggots are produce a cocktail of protease enzymes and antimicrobial compounds called ES [25],

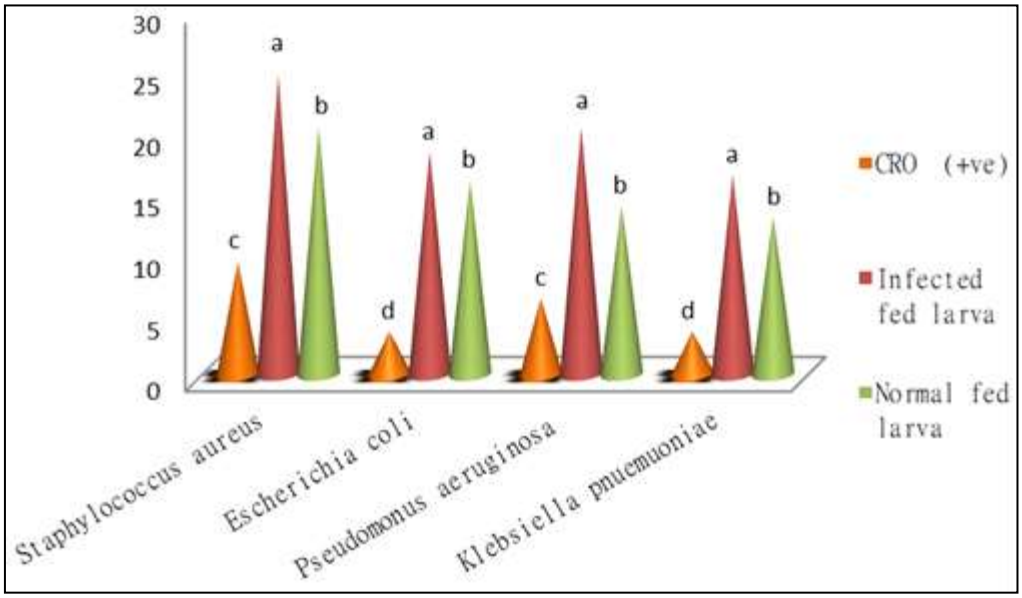

Fig 1: Relative antimicrobial activity of clear phase of larval SE fractionation of normal and infected fedlarvae of *Calliphora vomitoria* at 0.05 mg/ml

Conclusion

The excretions / secretions (ES) of the carrion blue bottle fly, *Calliphora vomitoria* L. larvae were inhibited growth of the tested sensitive Gram – positive and Gram – negatives bacteria; *Staphylococcus aureus, Escherichia coli, Pseudomonus aeruginosa* and *Klebsiella puenmoniae*. Also, ES inhibited growth of those previous bacterial species resistant piperacillin, and significantly that found better than standard drug (Ceftriaxone). The ES of the normal and others cannibalized infected and contaminated pathogenic larvae will encourage for more research in this topic.

Acknowledgment

The authors are grateful Mosul university authorities with anticipation to President of the university Prof. Dr. Kossay Alahmady for scientific support the first author. Also, thankful to Head of Biology Department; Assist. Prof. Dr. Mohmaad S. Faisal, for providing the research facilities in the Entomology laboratory/College of Education and Pure Sciences, we thank the Bacteriologist Dr. Alaa Taha Younis for diligent help in practical part of this article.

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