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Antibacterial activity of extracted hemolymph from larvae and pupae of local fly species, *Musca domestica* and *Chrysomya megacephala*

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ABSTRACT

Natural peptides in insect vectors played an important role in the control of pathogens. Musca domestica Linnaeus and Chrysomya megacephala Fabricius were two species of local fly chosen to detect presence of antimicrobial peptide substance. The screening of the antimicrobial activity was carried using a spectrophotometric method. Results were obtained much quicker and less laborious. The results showed larva hemolymph of M. domestica lysed Bacillus subtilis and two Gram negatives, Escherichia coli and Pseudomona. aeruginosa. The pupae hemolymph only lysed E. coli. Whereas, the hemolymph of C. megacephala larva showed bactericidal effect against both of the Gram positives tested, i.e. B. subtilis and Staph. aureus. and no effect was against the Gram negatives. The pupa showed lytic activity against Staph. aureus and P. aeruginosa. As a conclusion, the larva and pupa hemolymph of M. domestica and C. megacephala demonstrated antibacterial activity. However, larva hemolymph of M. domestica and C. megacephala has broader antibacterial activity against both Gram positive and negative bacteria.

Key words : Antimicrobial, insects, Musca domestica, Chrysomya megacephala, larva, pupae bactericidal effect, lyses, spectrophotometric method.

ABSTRAK

Kemunculan bakteria yang rintang terhadap antibiotik telah memangkinkan usaha mencari dan memencilkan antimikrob yang baru khasnya daripada sumber serangga. Musca domestica dan Chrysomya megacephala adalah dua sepsis lalat yang telah di kesan akan kehadiran bahan antimikrob ini. Kaedah penyaringan telah dilakukan dengan menggunakan kaedah spektrofotometer. Keputusan yang di dapati adalah sangat cepat dan mudah kaedahnya. Hasil kajian daripada hemolimfa larva Musca domestica mendapai ia mampu menglisiskan B. subtilis and dua Gram negatif, E. coli dan P. aeruginosa. Pupa serangga ini hanya mampu menglisikan E. coli sahaja. Manakala untuk hemolimfa larva Chrysomya megacephala, ia mampu menglisikan kedua-dua Gram positif yang digunakan tetapi tidak pada Gram negatif. Walau bagaimanapun pupanya aktiviti yang lebih bercampur di mana kesan litik berlaku terhadap bacteria Staph. aureus dan P. aeruginosa. Sebagai kesimpulannya

spertrum antimikrob yang luas telah didapati pada hemolimfa larva Musca domestica dan pupa Chrysomya megacephala.

Kata kunci: Antimikrobial, serangga, Musca domestica, Chrysomya megacephala, larva, pupa, kesan bakterisidal, lisis, kaedah spektrofotometrik.

INTRODUCTION

Recently the quest for the next generation of antibiotics is focused on natural peptides produced by animals or insects that ward of infections. The main reason behind the upsurge of interest is the advances of gene techniques have made antimicrobial peptide more accessible to study and production. In addition, the wide spectrum of activities reported for these amazing molecules suggests their potential benefits in treatments against viral infections (Balzarini et al. 2006), parasites (Hu and Aksoy 2006) and cancer (Brunel et al. 2005).

Insects are known for their ability to resist infection. They protect themselves against bacterial infection by secreting a battery of antimicrobial peptides into the hemolymph. Hemolymph, also known as the insect blood, is a clear fluid, with or without yellow or greenish pigmentation. It constitutes 16-40% of the body weight of certain insects. The volume and component of hemolymph are different among types of insects and their developmental stages. It spends much of its time flowing freely within body cavities where it makes direct contact with all internal tissues and organs. Therefore the circulation would help to transport the antimicrobial peptide to its target site (Kurata 2006).

In insect vectors, antimicrobial peptides may play a role in the control of gut pathogens. For example a sand fly *Phlebotomus duboscqi* was analyzed for its production of antimicrobial (Boulanger et al. 2004). After challenged by injected bacteria or feeding the sand flies with bacteria or the protozoan parasite *Leishmania major*, a new hemolymph peptide with antimicrobial activity was detected The peptide was identified to be a member of the insect defensin family. Interestingly, this defensin exhibits an antiparasitic activity against the promastigote forms of *L. major*, which reside normally within the sand fly midgut. *P. duboscqi* defensin could be induced by hemolymph or gut infections, or both (Boulanger et al. 2004). This means that the secretion of antimicrobial peptides into the gut may also depends on the microorganisms they ingested.

The objective was to investigate the presence of antimicrobial peptide of an unchallenged larvae and pupae of two species of house flies, *Musca domestica* and *Chrysomya megacephala*. Because of these two flies would usually dwell in an unhygienic environment, it is expected that they have already been exposed to many types of bacteria naturally. Therefore these insects in their early stage of development has developed some defenses against pathogens, including the antimicrobial peptides in the hemolymph.

MATERIALS AND METHODS

HEMOLYMPH EXTRACTION FROM LARVAE AND PUPAE OF *Musca domestica* AND *Chrysomya megacephala*

LARVAE HEMOLYMPH COLLECTION

Hemolymph was collected from pupae by clipping the anterior end near the cephalopharyngeal sekleton. The abdomen may have to be squeezed gently to get hemolymph to flow from the wound. During the whole process, the insect was chilled on ice prior to collection.

PUPA HEMOLYMPH COLLECTION

The puparium was clipped approximately a milimeter or so from the anterior of the puparium.

PROCESSING OF HEMOLYMPH COLLECTED

Hemolymph of the larvae and pupae were collected in sterile appendorf tube and maintained at or below 4^{o}C. 50µg of phenylthiourea (PTU) crystals was quickly added into the tube containing hemolymph. The phenylthiourea will inhibit the action of phenyloxidase in the insect hemolymph and prevent the hemolymph from turning black (melanizing). The hemolymph was quickly frozen for storage at -70 ^{o}C. or otherwise centrifuged at 1000 x *g* for 5 minutes at 4^{o}C before being used. This centrifugation step gives cell-free hemolymph; the hemocytes (blood cells) and any undissolved PTU crystals will be pelleted.

BACTERIA

Four species of the ATCC bacteria were used in this study. Two species were Gram positives *(Staphylococcus aureus Bacillus subtilis*) and the other two were Gram negatives (*Escherichia coli, Pseudomonas aeruginosa*).

POST ANTI MICROBIAL EFFECT (PAE) DETERMINATION

The PAE method used in this experiment was a modification of Dominguez et al. (2001). Two to three colonies of a 18 hour culture on nutrient were suspended in 50 mL prewarmed (37°C) Mueller–Hinton broth. The suspension was incubated overnight at 37°C, diluted 1/2500 in the same prewarmed medium and incubated in a waterbath at 37°C with agitation (50 rpm). The absorbance of the culture was monitored with a spectrophotometer (Shimadzu, Japan) at a wavelength of 660 nm until an absorbance of 0.1 was reached (equivalent to 2.5–3.0 x 10^7 colony forming unit(cfu)/ml for *E. coli* and *P. aeruginosa* and to 1.8–2.0 x 10^7 cfu/mL for *S. aureus*). Equal volumes of the control culture and the cultures to be treated with the

antibacterial hemolymph were prepared separately. The bacteria–antibacterial hemoymph contact lasted 1 hour. During the period, samples were taken at every 10 minutes.for the determination of bacterial concentration. Antimicrobial activity was stopped by placing a 10^{-3} dilution of the bacterial suspension in prewarmed Mueller–Hinton broth.

MATHEMATICAL FORMULATION TO DETERMINE BACTERIAL LYSES

The larva or pupae hemolymph naturally are yellow or greenish in colour. This colouration give a higher OD valued, compared to the control (bacteria suspension without hemolymph). Therefore, OD values for treated sample was always higher then the control and any bacterial lysis activity would be impossible to be detected. An adjustment was made to overcome the problem by removing the OD value of the hemolymph colouration. The formula for bacterial lyses activity would be,

ODa*n* or ODa*10* (example at 10 minutes) :

$$= [\text{ODhts}10 - (\text{Odhwbs} + \text{ODhts at time } 0 \times 10^3)/2] \qquad (1)$$

where OD = optical density value, ODa*n* = adjusted optical density at time *n* with *n* = the time where samples were taken, ODhts*n* = hemolymph treated sample (hemolymph with bacterial suspension), ODhwbs = hemolymph without bacterial suspension, ODhts at time 0 = hemolymph treated sample at time 0. This OD value were recorded immediately after hemolymph were added into the suspension which activity was stopped by placing a 10^{-3} dilution of the prewarmed Mueller – Hinton broth.

RESULTS

Two controls were used. One was the negative control, which was the bacterial suspension without hemolymph, and the positive control, bacteria suspension with 20mg/ml of gentamicin. Figs. 1 and 2 showed that the negative control has an almost plateau OD reading of 0.5. The number of bacteria increased slightly during the period of 1 hour to approximately 0.55. On the other hand, the positive control where the bacteria was exposed to Gentamicin demonstrated the bactericidal effect. The OD reading decreased from 0.5 down to 0.4 or more approximately around 0.3 as seen with *Bacillus subtilis* (Fig. 1). Figs. 1 and 2 showed the effect of *Musca domestica* larva and pupae hemolymph against two species of Gram positive bacteria, St*aphylococcus aureus* or *Bacillus subtilis*. With *Musca domestica* larvae hemolymph demonstrated bactericidal activity against *B. subtilis* (Fig. 1). The OD value started with 0.5 and gradually decreased during the period of 1 hour to approximately 0.35 in 50 minutes. However, the hemolymph from pupae did not show any effect against *B. subtilis*. Its OD readings were similar to the negative control. *Musca domestica*

hemolymph larvae and pupae has also failed to show any bactericidal activity against *Staph. aureus* (Fig. 2).

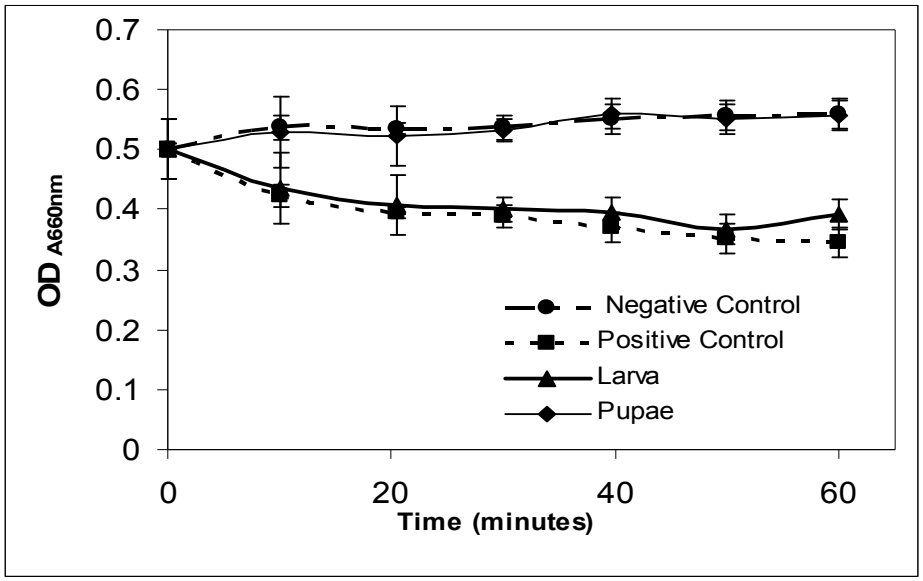

FIGURE 1 Effect of hemolymph from larva and pupae of from <u>*M. domestica*</u> against *B. subtilis*. Positive control represents Gentamicin (20mg/ml) with *B. subtilis*

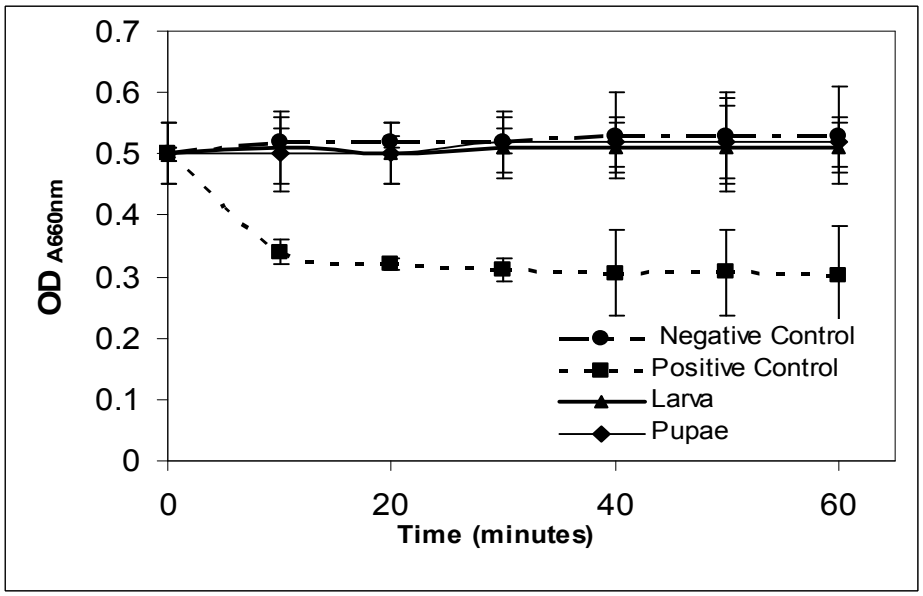

FIGURE 2 Effect of hemolymph from larva and pupae of from *M. domestica* against *Staph. aureus*. Positive control represents Gentamicin (20mg/ml) with *Staph. Aureus*

In Fig. 3, the larva and pupae of *Musca domestica* showed bactericidal effect against *E. coli*. The OD has decreased from 0.5 to approximately 0.45. In figure 4, the pupae hemolymph showed significant bactericidal effect against *P. aeruginosa*. The OD reading decreased very much lower then that compare to the positive control, approximately below 0.3. The larvae hemolymph also showed some slightly decreased pattern although its OD reading is slightly below the negative control line. *Chrysomya megacephala* larva and pupae demonstrated their activity against the two group of bacteria (Figs. 5, 6 7 and 8). The positive and negative control remained quite similar OD pattern as seen in the previous experiments. In figure 5, The larva hemolymph of *Chrysomya megacephala* demonstrated its bactericidal effect against *B. subtilis*, OD from 0.5 to approximately 0.43. Pupae hemolymph also recorded similar effect but the activity seems better than that of larvae. The OD has decreased to 0.4 within 20 minutes of exposure but increased slightly after 20 minutes. The bactericidal effect against *Staph. aureus* was detected with the larva hemolymph. The OD reading remained below the negative control after 10 minutes of exposure. Figure 7and 8 showed the *Chrysomya megacephala* larva and pupae hemolymph against two Gram negatives. Although larva hemolymph has not showed any bactericidal effect on *E.coli*, the pupae has showed significant effect. *P. aeruginosa* pupae hemolymph showed significant bactericidal effect. It has a decreased OD reading and was nearly similar to the positive control OD during the 20^{th} and 30^{th} minutes.

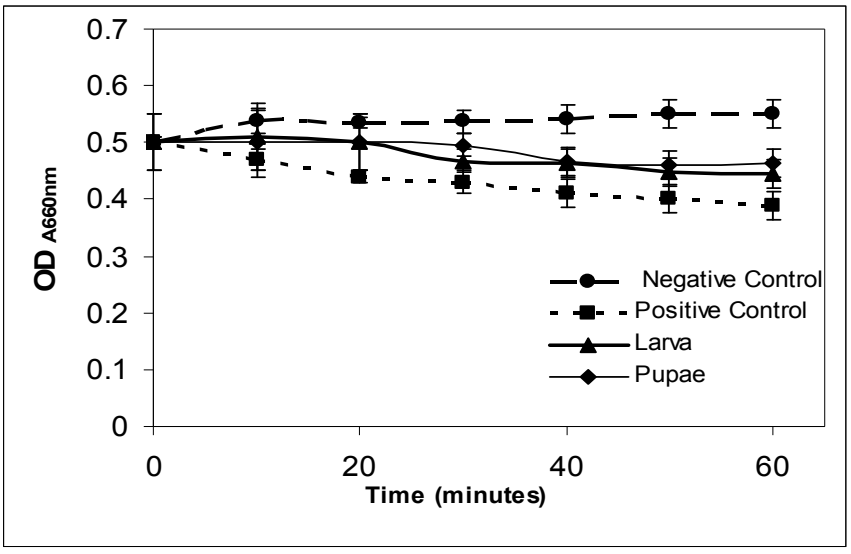

FIGURE 3 Effect of hemolymph from larva and pupae of from *M. domestica* against *E.coli*. Positive control represents Gentamicin (20mg/ml) with *E.coli*.

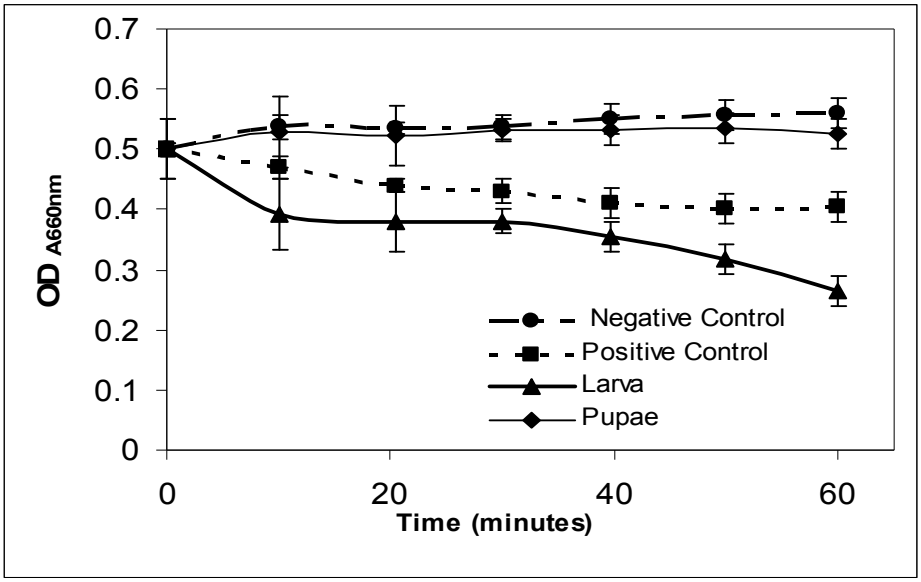

FIGURE 4 Effect of hemolymph from larva and pupae of from *M. domestica* against *Pseudomonas aeruginosa*. Positive control represents Gentamicin (20mg/ml) with *P. aeruginosa*.

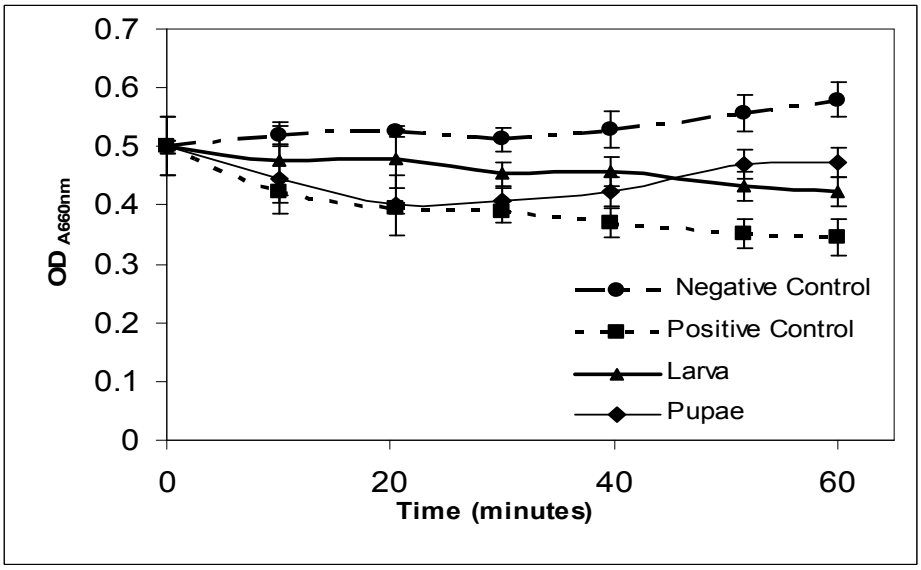

FIGURE 5 Effect of hemolymph from larva and pupae of *Chrysomya megacephala* against *B. subtilis*. Positive control represents Gentamicin

(20mg/ml) with *B. subtilis*.

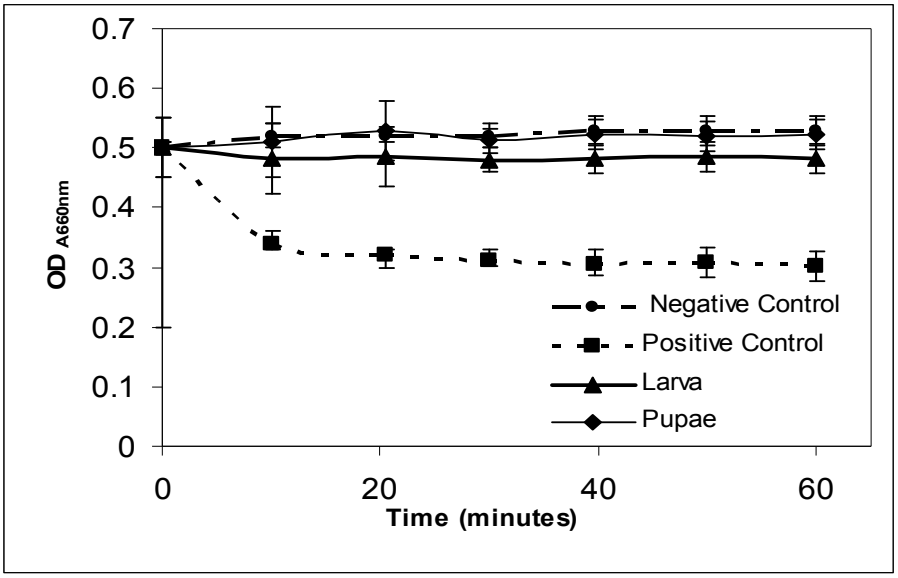

FIGURE 6 Effect of hemolymph from larva and pupae of *Chrysomya megacephala* against against *Staph. aureus*. Positive control represents Gentamicin (20mg/ml) with *Staph. aureus*.

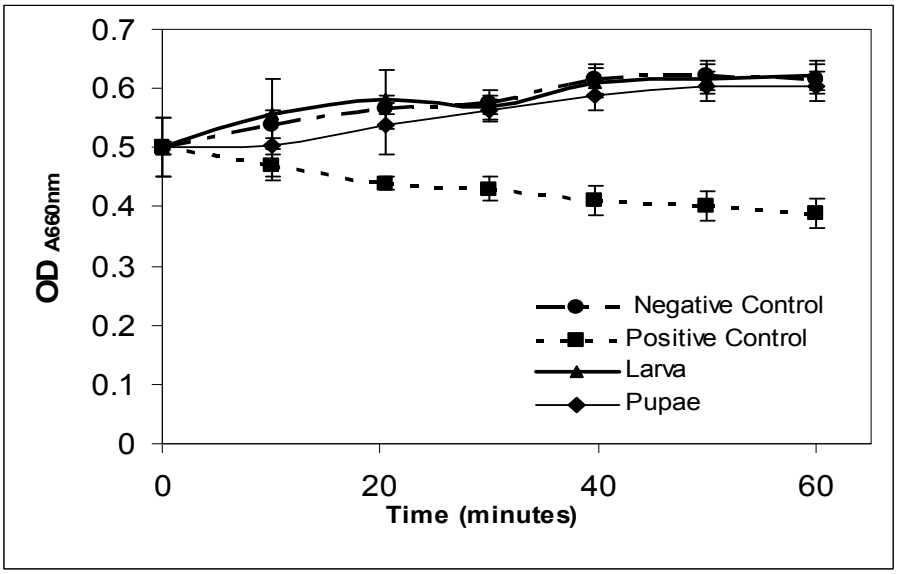

FIGURE 7 Effect of hemolymph from larva and pupae of *Chrysomya megacephala* against *E.coli*. Positive control represents Gentamicin (20mg/ml) with *E.coli.*

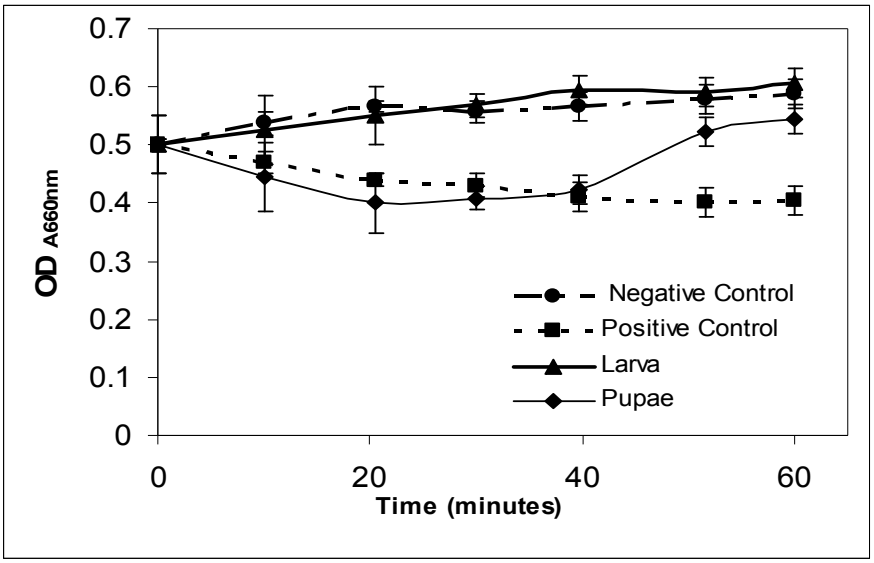

FIGURE 8 Effect of hemolymph from larva and pupae of *Chrysomya megacephala* against *Pseudomonas aeruginosa*. Positive control represents Gentamicin (20mg/ml) with *Pseudomonas aeruginosa*.

DISCUSSION

The spectrophotometric method employed was quicker, easier to use and to replicate. Its application in studying post-antibiotic effects has showed some significant usage. Similar quality was obtained when monitoring the growth kinetics by classic method of viable counts on agar plate compared to the spectrophotometer (Domínguez 2001). The counting on the plate method is much more laborious, slower and not cost effective enough.

Studies on insect's immune response had been carried out since early 1900. At presence, the study is more focused on its application towards human infections and diseases. *Musca domestica* and *Chrysomya megacephala* are two local species that are widely distributed in Malaysia (Sallehudin Sulaiman 1990). They are easily bred and propagated in the laboratory (Arbain 1990). Its ability to dominate the population of flies may depend on the survivability and adaptation in the environment especially to ward off infection.

In this study, the hemolymph from *Musca domestica* larvae has more success with *B. subtilis* and two Gram negative bacteria, *E. coli* and *P. aeruginosa* compared to pupae hemolymph, which managed to lyses *E. coli*.

The antibacterial activity of *Chrysomya megacephala* has also showed some promising results. Its larva hemolymph showed bactericidal effect against the two

Gram positive bacteria i.e. *B. subtilis* and *Staph. aureus*. No bactericidal effect was detected against the Gram negative bacteria in this study

The pupae hemolymph showed lytic activity against *Staph. aureus* and *P. aeruginosa*. *E. coli* however suggests the characteristics of the graph line produced a bacteriostatic effect instead. More investigation are needed to confirm this. In conclusion, a wide spectrum of bactericidal activity were showed by *Musca domestica* larva hemolymph and *Chrysomya megacephala* pupae hemolymph.

The nature of this bioactive molecules is not known. Although the presence of peptide molecule was not investigated in this study. Peptide may played the defense role in the early stage of development of *Musca domestica* and *Chrysomya megacephala* against bacteria. Further investigation are needed namely to isolate and characterized the compound.

CONCLUSION

Overall, the larva and pupa hemplymph of *M. domestica* and *C. megacephala* demonstrated antibacterial activity. However, larva hemolymph of *M. domestica* and *C. megacephala* has broader antibacterial activity against both Gram positive and negative bacteria.

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