LARVICIDAL EFFECT OF *METARHIZIUM ANISOPLIAE* CONIDIA AGAINST *AEDES ALBOPICTUS*

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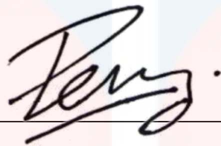
CERTIFICATION

This is to certify that we have read this research paper entitled **'Larvicidal effect of *Metarhizium anisopliae* conidia against *Aedes albopictus*"** by Nurainun Sofea Najwa binti Hairun. In our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 – Research Project.



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DEDICATIONS

I want to dedicate my work to my family and friends. Special thanks to my lovely grandparents, Hussain bin Abdullah and Hazizah binti Othman, who encourage and provided strength throughout this phase of work and lastly, my mother, Aznishah binti Hasan, a special thanks for always being there for me.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 5436 – Research Project.

Aedes albopictus is a mosquito species that can be commonly found in Malaysia. Study shows that *Ae. albopictus* can act as a vector that can potentially transmit dangerous diseases such as dengue and heartworm. Thus, chemical control was made to combat the increase in the population of mosquitoes. However, due to increased reports of resistance and toxicity effects, new control methods have been developed to replace the negative effect. Several studies show that entomopathogenic fungi such as *Metarhizium anisopliae* can be used as a biological control against *Ae. albopictus*. Thus, this study was conducted to determine the efficacy of *M. anisopliae* against *Ae. albopictus*. Secondary cultures of *M. anisopliae* were performed to collect the conidial suspension. A larvicidal bioassay based on the WHO standard was conducted with different treatment concentrations (10^1,10^3 and 10^5 conidial/ml). The observations were recorded for seven days, and the data were analysed using ANOVA and Probit analysis. The median lethal concentration (LC_{50}) and lethal concentration 90 (LC_{90}) for the conidia suspension were 9.93×10^2 conidia/ml and 1.5×10^4 respectively. Whereas the median lethal time 50 (LT_{50}) for the concentrations of 10^1, 10^3 and 10^5 are 14.05 days, 13.24 days, and 2.20 days and the median lethal time (LT90) is 25.6 days, 23.7 days and 3.3 days. The ANOVA result shows that there are no significant different between the concentrations of treatments and larval motility with P value > 0.05. However, this study proves that *M. anisopliae* has an effective effect against *Ae. albopictus larvae* which could be developed as a potential bio-insecticide for *Ae. albopictus control.*

Keywords*: Aedes albopictus, Metarhizium anisopliae*, biological control, larvicidal bioassay.

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ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Aedes albopictus adalah spesies nyamuk penting yang biasa ditemui di Malaysia. Kajian menunjukkan bahawa *Ae. albopictus* boleh bertindak sebagai vektor yang berpotensi untuk menularkan penyakit berbahaya seperti denggi dan cacing hati. Justeru itu, kawalan kimia dilakukan bagi membanteras pertambahan populasi nyamuk ini. Walau bagaimanapun, disebabkan peningkatan laporan terhadap rintangan dan kesan toksik, pelbagai kaedah kawalan baru telah dibangunkan untuk menggantikan kesan negatif tersebut. Beberapa kajian menunjukkan kulat entomopatogen seperti *Metarhizium anisopliae* boleh digunakan sebagai kawalan biologi terhadap *Ae. albopictus*. Justeru itu, kajian ini dijalankan untuk menentukan keberkesanan *M. anisopliae* terhadap *Ae. albopictus*. Kultur sekunder *M. anisopliae* telah dikumpulkan bagi memperolehi konida. Bioassay larvisidal telah dijalankan berdasarkan rujukan WHO dengan kepekatan rawatan yang berbeza (10^1, 10^3 dan 10^5 konidia/ml). Pemerhatian direkodkan selama tujuh hari, dan data analisis dijalankan menggunakan analisis ANOVA dan Probit. Kepekatan maut 50 (LC_{50}) dan kepekatan maut 90 (LC_{90}) untuk konidia ialah $9.93x10^2$ konidia/ml dan $1.5x10^4$. Manakala, masa kematian bagi LT_{50} bagi setiap kepekatan 10^1, 10^3 and 10^5 adalah 14.05 hari, 13.24 hari, dan 2.20 hari dan masa kematian LT_{90} pula adalah 25.6 hari, 23.7 hari dan 3.3 hari. Keputusan ANOVA menunjukkan bahawa tidak terdapat perbezaan yang signifikan bagi kepekatan berbeza dan kematian dengan nilai $P>0.05$. Walaubagaimanapun, kajian ini membuktikan bahawa *Metarhizium anisopliae* mempunyai kesan terhadap *Ae. albopictus* larva dimana ia berpotensi untuk menjadi racun perosak bio untuk kawalan *Ae. albopictus*.

Kata kunci*: Aedes albopictus, Metarhizium anisopliae*, kawalan biologi, bioassay larvisida.

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1.0 Introduction

Aedes albopictus is one of the vital mosquito species that can be found in Malaysia. It belongs to the class of Insecta and order of Diptera. Study shows that *Ae. albopictus* originated from Asia and specialised in colonising temperate environments (Gratz, 2004). Furthermore, these mosquitoes are vectors that can potentially transmit dangerous viral diseases such as dengue and Rift Valley Fever (Choi et al., 2020). The present control for *Aedes* spp. nowadays is still heavily dependent on chemical pesticides. These chemical pesticides are known for their toxic effect on humans and animals.

There are increasing reports of *Aedes* spp. developing resistance towards chemical pesticides., potentially leading to a higher *Aedes* spp. population and an increase in viral disease transmission. A new method has been applied to controlling these vectors as biological pesticides. The usage of biological pesticides would reduce our dependence on chemical pesticides.

Metarhizium anisopliae is an entomopathogenic fungus that can be found within the environment, such as soil. It belongs to the class of Sordariomycetes and the order of Hypocreales. The most used forms of *M. anisopliae* for biological pesticides are conidia and blastopore. Some studies have shown that conidia appear to be more virulent than blastospores but some stated that the virulency is the same dependence on the mosquito species tested (Alkhaibari et al., 2017)

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These conidia have the ability to germinate and develop hyphae that are able to penetrate cuticles in suitable hosts such as mosquitoes larvae leading to cascades of recognition and enzymatic reaction (Aw & Hue, 2017). Upon propagation within the larvae, it would present the fungal potential for inoculum to amplify within the host. The conidia consist of two forms which are wet and dry and studies show that the dry form is more virulent than the wet form. Moreover, conidia appear to be more persistent and virulent as they display better protection against UV radiation, fluctuating temperature and humidity (Aw & Hue, 2017). The conidia also have the ability to be transmitted from one mosquito to another in a population through the mating of male mosquitoes that are infected with conidia with intact female mosquitoes (Reyes-Villanueva et al., 2011)

Furthermore, these fungi have the ability to destroy these vectors without leading to any chemical pollution like chemical pesticides. Upon the death of the infected vectors, it would emerge and produce sporulation on the cadaver. Thus, producing new opportunities for the fungus to infect new vectors. As an alternative to chemical pesticides, *M. anisopliae* comprises a potential source of bioactive compounds and is generally free of harmful effects (Litwin et al., 2020).

This study was conducted to identify and determine the efficacy of *M. anisopliae* against *Ae. albopictus.* It was conducted to discover the biological potential of the larvicidal effect of the fungus against *Ae. albopictus*. Moreover, the local isolated *M. anisopliae* has a good prospect of becoming an efficient mosquito or vector control in Malaysia.

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2.0 Problem Statement

- *Metarhizium anisopliae* is a well-known entomopathogenic fungus used especially against pest control. The virulency of this fungus is usually dependant on the selection of the pathogenic strain with specific efficacy of the selected target hosts such as *Aedes* spp. However, there is limited research on determining the larvicidal effect of *M. anisopliae* conidia against *Ae. albopictus* in Malaysia.

3.0 Justification

- This study is conducted to discover an alternative source and safer method for the development of biological pesticides towards *Ae. albopictus* in Malaysia.

4.0 Research Questions

- What is the efficacy of *M. anisopliae* conidia against *Ae. albopictus*?
- What is the lethal concentration lethal time of *M. Anisopliae* conidia against *Ae. albopictus* for LC_{50}, LC_{90}, LT_{50} and LT_{90}?

5.0 Research Hypothesis

- *Metarhizium anisopliae* conidia are highly efficacious against *Ae. albopictus*. It is pathogenic to *Ae. albopictus* larvae, providing a greater window of control of these vectors.

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6.0 Objectives

- To determine the efficacy of *M. anisopliae* conidia against *Ae. albopictus*
- To determine the lethal concentration and lethal time of *M. anisopliae* conidia against *Ae. albopictus* for LC_{50}, LC_{90}, LT_{50} and LT_{90}.

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7.0 Literature review

7.1 Aedes albopictus

Mosquitoes consist of several genera such as *Aedes*, *Anopheles* and *Culex*. They are blood-sucking arthropod vectors of humans and animal diseases worldwide. *Aedes albopictus* was reported as the vector or mediator for dengue haemorrhagic fever, yellow fever virus, Chikungunya, Zika and West Nile viruses (Choi et al., 2020). They tend to search for humans but can also bite animals. *Aedes albopictus* has the mannerism of biting diverse host species thus enabling them to become a potential bridge for certain pathogens that are multi-host.

Aedes albopictus or known as *Stegomyia albopicta* or Asian tiger mosquito is a native mosquito located in tropical and subtropical areas. Over the last two decades, *Ae. albopictus* has spread from western Pacific and Southeast Asia to Europe, Africa and others through the transport of goods and travels (Gratz, 2004).

7.2 Mosquito concern and alternative control

Aedes albopictus is one of the mosquito species that are very difficult to suppress or control due to their ability in adapting to various environments and their reproductive biology. Mosquito control has mainly been dependent on chemical insecticides. However, chemical insecticides have many toxic effects on humans and animals. There are increasing reports of resistance in mosquito populations against insecticides (Vontas et al., 2010, Rasli, 2021). To combat this resistance, various control methods such as environmental and biological control have been developed to replace the negative effects (Benelli et al., 2016). A recent study has proposed that biological control by using entomopathogenic fungi can be used as

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an effective alternative to control the larval stages of the mosquitoes (Buckner et al., 2017). *Metarhizium anisopliae* is one of the most commonly used entomopathogenic fungi for pest control (Lacey et al., 2015). However, there was lack of case studies done for understanding the biological effects of the local isolated *M. anisopliae* against *Ae. albopictus.*

7.3 *Metarhizium anisopliae* conidia and pathogenicity

Metarhizium anisopliae is a fungus that grows in the environment such as soils. It is a mitosporic fungus with asexual reproduction and was classified in the form class of Hyphomycetes of phylum Deutromycota (Shinkafi & Sanusi, 2013). *Metarhizium anisopliae* is commonly used due to its environmental friendliness and ease of mass production. There are several common forms of fungi which are blastospores and conidia. The conidia would adhere to the epicuticle of the host. The outer layer of conidia contains hydrophobins which help in facilitating the adhesion. These would lead to the germination of the conidia as it will be initiated with the presence of carbon and nitrogen sources. After germination, the spores would swell and produce germ tubes and subsequently appressorium formation. It will secrete a thin layer of mucilage to consolidate the attachment of the fungus. Thus, leads to the penetration stage which will lead to the death of the host. *Metarhizium anisopliae* also has the ability to initiate the expression of genes for rapid multiplication within the host. During sporulation, the *M. anisopliae* hyphae would extrude the host cuticle to the outer environment (Aw & Hue, 2017).

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8.0 Materials and Methods

8.1 Fungal culture

The primary culture of *Metarhizium anisopliae* (HSAH5) was obtained from Universiti Putra Malaysia. The culture was inoculated on mosquitoes and was isolated from the mosquito cadaver before culturing. The fungus was grown on SDA media and maintained under dark conditions with a room temperature of 28 ± 2 °C until grown. A secondary culture was performed on the SDA media with a streaking method of zig zag. The fungus was sealed with parafilm and was incubated until full conidia formation. The fungus was left for two weeks to observe the growths at room temperature of 28 ± 2°C as shown on figure 8.1.

Figure 8.1: Secondary culture of Metarhizium anisopliae at 14 days of observations

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8.2 Mosquito source and maintenance

The WHO standard on mosquito rearing and maintenance was used in this study (WHO, 2015). The eggs of *Ae. albopictus* (laboratory strain) were obtained from Universiti Sains Malaysia. The eggs were put into shallow trays filled with tap water for incubation. Bacterial growth de-oxygenated the water, triggered the egg hatching, and induced the first instar to hatch within 12 hours of dehydration. The larvae were fed with fish food (tetra pro) and managed at room temperature of 28 ± 2 °C, in 16L:8D photoperiod. The amount of food was kept low at first and gradually increase as the larvae grow. The feeds were given severally at intervals of 1 to 2 days. A homogenous population of late 3rd or early 4th instars was obtained within two to three days as shown in figure 8.2. Daily observations of the larvae were required.

Figure 8.2: Homogenous population of late 3rd to early 4th instar of Aedes albopictus

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8.3 Conidial suspension

Fresh conidia were harvested from the secondary culture by adding 10ml sterile distilled water with 0.1% Tween 80 in a test tube and the agar was scraped with a sterile spatula to dislodge the conidia. Three to five plates were used to collect the conidia needed for the bioassay. The collected suspension was vortexed for 5 minutes to ensure homogenization. This suspension was filtered through sterilized filter paper to remove mycelia as shown in figure 8.3. The conidia suspension concentration was counted in the hemocytometer chamber and viewed under the microscope.

Figure 8.3: Conidial suspension of Metarhizium anisopliae

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8.4 Larvicidal Bioassay

Ten of the 3rd or 4th instar larva were transferred by using a dropper to a weighing boat containing a small amount of water from the primary rearing tray of the mosquitoes. The small, unhealthy or damaged larvae were removed. The depth of water in the cups was remained between 5-10cm to avoid undue mortality. A suspension concentration of 10^7 conidial/ml was prepared. Serial dilutions were prepared for subsequent concentrations (10^1, 10^3 and 10^5 conidial/ml). Three replicates were set up for each concentration and an equal number of controls were set up simultaneously with distilled water + 0.1% Tween 80 as shown in figure 8.4. The serial dilutions were added first in the cups, followed by the insertion of the larvae.

Figure 8.4: Larvicidal bioassay of Metarhizium anisopliae

8.5 Larval Motility Observation

The larval motility was observed every 24 hours intervals subsequently for seven days of observation. Death and moribund larvae were observed and detected. These results were recorded in Table 9.1. Abbot formula was applied in this study as the control mortality was between 5-20%. This formula was applied:

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$$\text{Mortality (\%)} = \frac{X - Y}{X} \times 100,$$

where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

The dead larvae will be observed under the electron microscope for further identification and observations. The treatment was repeated three times.

8.6 Data analysis

Analyses were done by using one way ANOVA and Probit analysis. Thus, allowing the determination of larvicidal effects at mean lethal concentration 50 (LC_{50}), lethal concentration 90 (LC_{90}), lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) of *M. anisopliae*.

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9.0 Results

On the first day of observations, the larvae within the highest concentration (10^5) appeared to have a reduction in the larvae activity compared to concentrations of 10^1 and 10^3. The larvae appear less motile and inactive compared to larvae in other concentrations of 10^1 and 10^3. These larvae show a reduced inability to rise to the water surface. There was less induction of movement and diving characteristics when the larvae were probed with the dropper. Whereas the reduction in movement and activity of the larvae within concentrations of 10^1 and 10^3 can only be observed on the third day of observations. By the fifth day of observations, the larvae within the highest concentration (10^5) have a total reduction in movement and no capability to rise to the water surface.

The larvae mortality results were recorded in Table 9.1. On day-1 of observations, there was the presence of moribund and dead larvae in the highest concentration (10^5) of the treatment at 2.5% compared to concentrations of 10^1 and 10^3. The larval mortality in larvae within concentrations of 10^1 and 10^3 can be observed on day-3 of observations at 0.75% and 4% respectively. Larval mortality of the control was observed on day-4 and 5 in which 17.7% and 18.9% of the control larvae were dead. Moreover, a 100% mortality was achieved by the highest concentration (10^5) at day 5. A comparison of the mortality percentage revealed that 10^5 has caused the highest mortality based on table 9.1.

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Table 9.1: The percentage (%) of moribund and dead larvae after corrected with Abbot formula

Day	Percentage (%) of moribund and death larvae in concentrations of treatment		
	10^1	10^3	10^5
1	0	0	2.5
2	0	0	42.5
3	0.75	4	77.5
4	-1.15	-3.28	93.92
5	6.60	5.36	100
6	12.68	28.06	100
7	26.02	33.11	100

Table 9.2: The cumulative percentage (%) of moribund and dead larvae in different concentrations of treatments

Concentrations of treatment	Cumulative percentage (%) of moribund and dead larvae	P-value
10^1	26.02	
10^3	33.11	$P > 0.05$
10^5	100	

A one-way ANOVA was conducted to determine the association of larval motility within different concentrations. Results in table 9.2 shown that there is no significant difference between concentrations of the treatments and larval mortality as the $P > 0.05$.

Whereas a Probit Analyses was conducted to determine the lethal concentration 50 (LC_{50}), lethal concentration 90 (LC_{90}), lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}). The lethal

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concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}) for the conidia suspension was 9.9×10^2 conidia/ml and 1.5×10^4 respectively. Whereas the median lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) for the concentrations of 10^5 were the shortest among different concentrations (10^1 and 10^3) at 2.20 days and 3.3 days. Meanwhile, the lowest concentration 10^1 has the longest lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) at 14.05 and 25.6 days as shown in table 9.3.

Table 9.3: Lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) of Aedes albopictus against Metarhizium anisopliae

Lethal time	Time taken to induce larval motility in different concentrations of treatment (days)		
	10^1	10^3	10^5
Lethal time 50 (LT_{50})	14.05	13.25	2.20
lethal time 90 (LT_{90})	25.6	23.7	3.3

After the larvae were moribund and died, the larvae were incubated in a petri dish at room temperature for final observations. The result shows that there were two stages of *Metarhizium anisopliae* development on the larvae cadaver. The first stages were the growth of white mycelia, and the second stages were the green discolouration of the mycelia indicating the presence of mature mycelia. The full growth of white mycelia on the larvae cadaver formed on the 3rd-day post mortality in the highest concentration (10^1) compared to concentrations of 10^1 and 10^3 as shown in figure 9 A. Whereas the green mycelia was started to grow at 5 days of post mortality in the highest concentration (10^1) as shown in figure 9 B.

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Figure 9.A: Mycelia growth of Metarhizium anisopliae from larvae cadaver from 3-day post mortality. (a) Absence of white mycelia formation on larvae cadaver in a concentration of 10^1. (b) Present of white mycelia at the head and upper part of the abdominal region of larvae cadaver in a concentration of 10^3 (c) Present of white mycelia covering the whole larvae cadaver on the concentration of 10^5

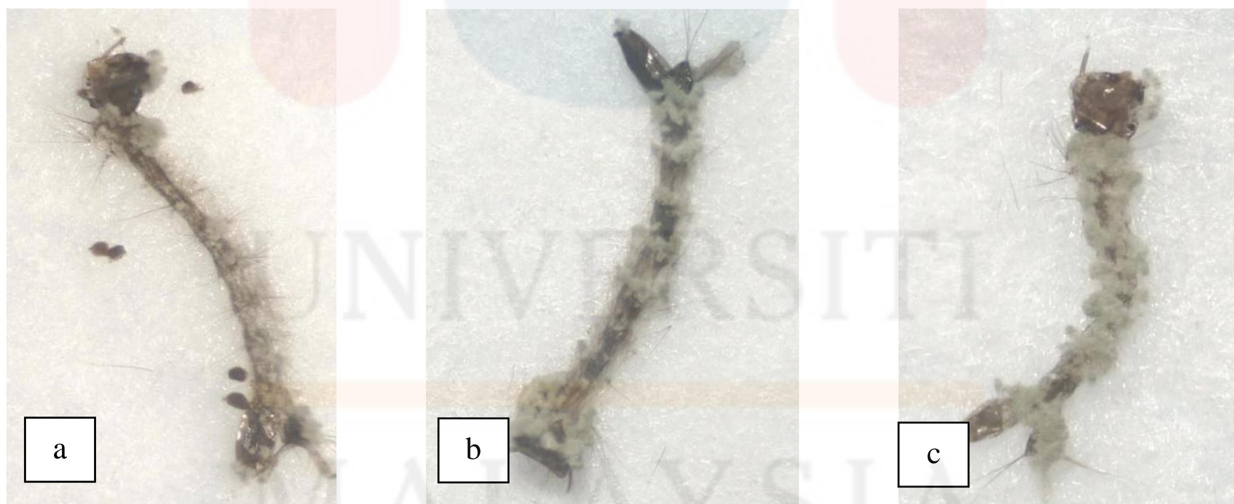

Figure 9 B: Mycelia growth of Metarhizium anisopliae on the larvae cadaver from 5-day post mortality. (a) Present of green mycelia covering the head and thorax region of the larvae cadaver on the concentration of 10^1. (b) Present green mycelia covering the head, thorax and abdominal part of the larvae cadaver at a concentration of 10^3 (c) Present green mycelia covering the whole region of the larvae cadaver at a concentration of 10^5.

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10.0 Discussions

All treated larvae within the study show reduction in the larvae activity with the highest concentrations being the most affected as there was an increase in conidial attachment on the larvae cuticle at the highest concentration. The conidia and hyphae formation at the integument would lead to penetration of muscle and nerve tissue rarification (Hänel, 1982). This would lead to disruption within the neuronal synapse and lead to a decrease in larval behaviour activity. Thus, producing decreased larvae activity such as sluggishness and failure to reach the water surface.

At the same time, the time of death is usually dependent on the infection dose. The highest mortality rate would usually be achieved in the highest concentrations where a 100% mortality was able to be achieved on day-5 observation for the highest concentration (10^5). A higher virulence rate is related to an increase in the adhesion of the conidia on the larvae cuticle. This occurs as there would be an increase in the probability of conidia attachment within the highest concentrations. Thus, leading to higher virulence and larvae mortality.

Furthermore, the ANOVA result shows that there is no significant association between the concentrations of conidial suspension and the larval mortality with a P-value >0.05. However, the larval mortality is usually dose dependent (Bilal et al., 2012, Zuharah et al., 2021)

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In this study, the lethal concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}) for the conidia suspension were $9.93x10^2$ conidia/ml and $1.5x10^4$ conidial/ml respectively. This result was higher compared to several case study where the lethal concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}) was achieved at $9.6x10^3$ and $1.2x10^5$ (Zuharah et al., 2021), $1.09x10^5$ and $1.9x10^{13}$ (Bilal et al., 2012) and $9.58x10^5$ and $1.18x10^7$ (Maldonado-Blanco et al., 2014)

This result revealed that only a lower concentration of conidial suspension is needed to achieve a high mortality rate in this study. This result is in agreement with a study by Zuharah et al. (2021) which shows there is only little significant difference in lethal concentrations needed to achieve the lethal concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}). However, the research from Bilal et al. (2012) and Maldonado-Blanco et al., (2014) shows significant differences in the lethal concentrations needed to achieve the larval mortality as the research works carried out in the temperate, and sub-tropical regions with their local fungal isolates might have different significance effect compared to tropical countries. The reason for this partly lies in the different pathogenicity of these fungus isolates as they vary by geographical conditions (Polovinko et al., 2010)

Besides, in this study, the LT_{50} and LT_{90} for the concentrations of 10^5 was the shortest which were achieved between 2.20 day to 3.3 days. This result showed that the highest concentration (10^5) would be effective in inducing larvae motility due to the life cycle of *Ae. albopictus*. *M. anisopliae* would be able to induce pathogenicity upon acting on the 3rd to early 4th instar of the larvae within 2-3 days. The highest concentrations would be able to kill the larvae before they moulted into the pupae stage. Whereas concentrations 10^1 and 10^3

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would not be able to induce larvae motility of the 3rd instar as it would take longer than 20 days to achieve the lethal time meanwhile *A. albopictus* would complete and achieve adult life cycle within 7 days.

Furthermore, the mortality rate of the control group in this study is between 17.7 to 18.9. According to World Health Organization (WHO), the accepted mortality rate of the control group is between 5-20% which is accepted in this case. Nevertheless, the mortalities of the treated group would need to be corrected by using the Abbot formula (WHOPES, 2005). The death of the control group occurs mostly due to the mishandling of the larvae. One of the mishandlings conducted was the lack of feed given to the larvae within the control group. The feed should be given at least once every 48 hours intervals to ensure the survival of the control group larvae (WHOPES, 2005).

The development of the mycelia formation on the larvae after death was confirmed that the larvae were dead due to the exposure to *M. anisopliae* (Hänel, 1982). These developments of the mycelia occur after the dead larvae were incubated at room temperature. Mycelial formation of *M. anisopliae* has been reported for their larvicidal, cellulolytic and cytotoxic activity (Ragavendran et al., 2019).

The formation of white mycelia occurs 3 days post-mortality, followed by the formation of green mycelia the 5 days post mortality. These findings correlated with a study that show that mycelia would form within 96-120 hours in which the dense mycelium formation would usually develop within the body cavity and the hyphae would gather at a certain area under the cuticle leading to surface sporulation (Hänel, 1982). The mycelia formation rate was

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also correlated with the concentration used as a higher concentration has visible mycelia formation in a relatively short period of time. The conidia were visible in all concentrations started on day-4 post mortality. However, the coverage of the conidia growth was higher in higher concentrations (10^5).

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11.0 Conclusions

In conclusion, *M. anisopliae* was proven to be effective against *Ae. albopictus* as it can induce the larval mortality, especially at the highest concentration of conidial suspension (10^5). Moreover, in this study, a lower value of LC_{50} and LC_{90} appeared to be virulence in inducing the larval motility of *Ae. albopictus* and an average of 2.2. to 3.3 days were only needed to induce the lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) which suggesting that the *M. anisopliae* strain used in this study has the potential to become bioinsecticide for controlling *Ae. Albopictus*.

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12.0 Recommendations and future work

There were several limitations observed in this study. For future reference, it is advisable to store the mosquito eggs at a suitable temperature and storage in order to induce hatchability of the eggs. It is also advisable to hatch the eggs in a dark coloured shallow tray as a bright colour shallow trays such as red and yellow would reduce the hatchability of these eggs. Moreover, the eggs need to be hatch as soon as possible. Avoid storing the eggs for two weeks as it would decrease the hatchability rate of the eggs. Improper storage and unsuitable temperature can lead to the death of the eggs before hatching. Furthermore, the environmental and nutritional factors in inducing the egg hatchability should be taken note of.

A further study is recommended to determine the efficacy of *M. anisopliae* pathogenicity in inducing larval and adult mortality of *Ae. albopictus*. A cross-species examination is recommended in order to observe the larvicidal effect of *M. anisopliae* against *Aedes sp.* and different fields and laboratory strains. A formulation for *M. anisopliae* needs to be studied in order to increase the efficacy against *Ae. albopictus* and further reduce the lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}).

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Appendices

Appendix A:1: First repetitions of larval mortality observations (5/4/2022)

Day	10^1	10^3	10^5	Control
1	0	0	1	0
2	0	0	10	0
3	0	0	40	0
4	0	0	40	0
5	0	0	40	0
6	0	0	40	0
7	0	0	40	0

Appendix A:2: Second repetitions of larval mortality observations (20/4/2022)

Day	10^1	10^3	10^5	Control
1	0	0	2	0
2	0	0	20	0
3	1	1	24	0
4	13	6	37	12
5	18	7	40	12
6	21	17	40	12
7	28	25	40	12

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Appendix A:3: Third repetitions of larval mortality observations (20/4/2022)

Day	10^1	10^3	10^5	Control
1	0	0	0	0
2	0	0	21	0
3	0	4	29	0
4	7	12	37	4
5	11	21	40	5
6	14	27	40	5
7	20	30	40	5

Appendix A:4: Average value (n) of larval mortality observations

Day	10^1	10^3	10^5	Control
1	0	0	1	0
2	0	0	17	0
3	0.3	1.6	31	0
4	6.7	6.0	38	5.3
5	9.7	9.3	40	5.67
6	11.67	14.67	40	5.67
7	16	18.3	40	5.67

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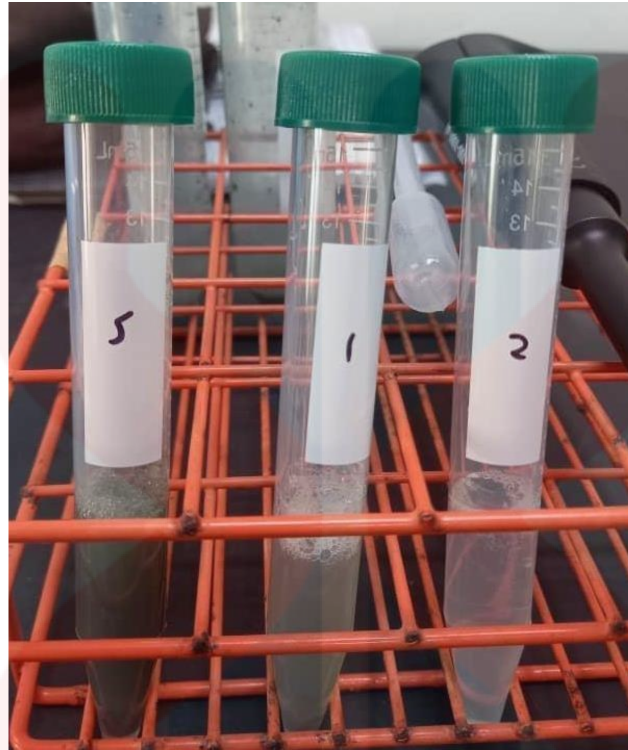

Figure A:1: Serial dilution of Metarhizium anisopliae

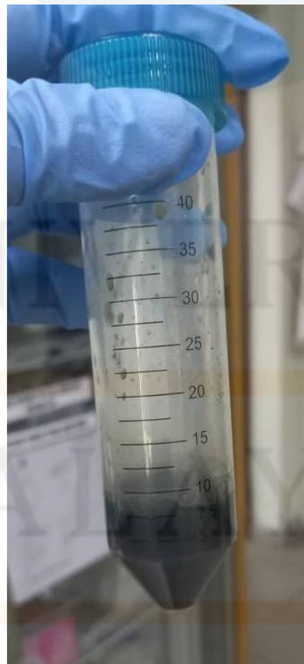

Figure A:2: Filtered conidial suspension of Metarhizium anisopliae

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