LARVICIDAL EFFECTS OF *METARHIZIUM ANISOPLIAE* BLASTOSPORE AGAINST *AEDES ALBOPICTUS*

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Larvicidal Effects of *Metarhizium Anisopliae Blastospore* against *Aedes Albopictus'*** by Muhammad Fahmi Bin Ramli. 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

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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 an important vector especially for the transmission of many viral pathogens, including dengue fever, Chikungunya fever and yellow fever (Motoki et al., 2019). Besides that, *Ae. albopictus* also can act as a vector that can highly transmit dangerous diseases such as heartworm that are common in dogs. In order to reduce the risk of infections, many integrated approaches have been done including chemical control methods by using insecticides. But due to over exposure towards the insecticides, resistance started to develop in the mosquitoes. Therefore, the aim of this research to determine the larvicidal effects of *Metarhizium anisopliae blastospore* against *Ae. albopictus* that later might become a successful alternative control. This research was conducted by expose *Ae. albopictus* larvae to different concentration of *M. anisopliae blastospore suspensions* (10^1, 10^3 and 10^5 blastospore/ml). The dead larvae were then observed for 7 days and the data were analysed using ANOVA and Probit analysis. The median lethal concentration 50 (LC_{50}) and lethal concentration 90 (LC_{90}) for the blastospore suspension was $5.6x10^4$ blastospore/ml and $7.5x10^6$ respectively. The results show that there is no significant difference in larval mortality between the concentrations used. Hence, this study might prove that *M. anisopliae* have an effective effect against *Ae. albopictus.*

Keywords: *Aedes albopictus, Metarhizium anisopliae* blastospore, *biological control* larvicidal effects

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 merupakan vektor penting terutamanya di dalam penyebaran patogen virus, termasuk demam denggi, demam Chikungunya dan, demam kuning. Selain itu, *Ae. albopictus* juga bertindak sebagai vektor yang boleh menularkan penyakit berbahaya seperti jangkitan cacing pada jantung yang biasa berlaku pada anjing. Oleh itu, untuk mengurangi risiko jangkitan ini, pelbagai pendekatan telah dilakukan termasuklah kaedah kawalan kimia dengan menggunakan racun serangga. Namun disebabkan kekerapan penggunaan racun serangga terhadap nyamuk, ianya mula beradaptasi dan mempunyai daya tahan terhadapnya. Tujuan penyelidikan ini dilakukan adalah untuk menentukan kesan larvisid *M. anisopliae* blastospore terhadap *Ae. albopictus* yang dimana ianya mungkin bakal menjadi kawalan alternatif yang berjaya. Penyelidikan ini dilakukan dengan memasukkan larva *Ae. albopictus* ke dalam ampaian *M. anisopliae* blastospore yang mempunya pelbagai kepekatan iaitu (10^1, 10^3 and 10^5 blastospore/ml). Larva yang telah mati akan diperhati selama tujuh hari menggunakan ANOVA dan Probit analisis. Kepekatan maut 50 (LC_{50}) dan kepekatan maut 90 (LC_{90}) untuk blastospore ialah pada $5.6x10^4$ blastospore/ml dan $7.5x10^6$. Keputusan menunjukkan bahawa terdapat perbezaan yang tidak signifikan bagi kematian larva. Oleh itu, kajian ini mungkin membuktikan bahawa *M. anisopliae* mempunyai kesan terhadap *Ae. albopictus.*

Kata kunci: *Aedes albopictus, Metarhizium anisopliae blastospore, Kawalan biologi, Kesan larvisid*

1.0 Introduction

Aedes albopictus is the dominant *Aedes* mosquito species and one of the main vectors that cause dengue fever in Malaysia. The global expansion of *Ae. albopictus* depends on several factors such as climate changes, geographical distribution, and human activities (ECDC, 2019). *Aedes albopictus* or also known as tiger mosquito is native to the tropical and subtropical areas of Southeast Asia. *Aedes albopictus* belongs to the class of Insecta, Order Diptera, and *Culicidae* family (ECDC, 2019).

There are many control strategies that have been done to manage the *Ae. albopictus* population in Malaysia and are mainly based on the use of insecticides. But nowadays resistance to these insecticides has occurred among the *Aedes sp.* (Accoti, 2021), due to being frequently exposed to these insecticides. In order to control the insecticides resistance problem in *Aedes sp*., researchers come up with an alternative development and at the same time eliminate the harmful impacts of insecticides toxicity with a new biological control method using *M. anisopliae*, which is a type of fungus that has a certain mode of infections on mosquitoes and midges.

Metarhizum anisopliae is a type of mitosporic fungus with asexual reproduction that grows naturally in soils throughout the world and causes disease in various insects by acting as an entomopathogenic fungus. *M. anisopliae* was formerly classified in the class of Hyphomycetes of the phylum Deuteromycota (Alkhaibari et al., 2018). *M. anisopliae* have their own specific mode of pathogenicity that requires the spores to adhere to the surface of the host cuticle and follow by the colonization of hemocoel. There are two forms of *M. anisopliae* which are conidia and blastospore form. Both of these forms have their own specific pathogenicity and are highly efficacious in order to kill the mosquitoes' larvae, but blastospores are generally considered more virulent

than conidia based on the mode of pathogenesis where blastospores appear to be more dependent on entry using mechanical force and specific enzyme secretion. Besides that, blastospores take about 24 hours to germinate faster than conidia and this also one of the factors that is considered to be attributed to its virulence determinant (Malassigné et al., 2020).

2.0 Problem Statement

2.1 *Metarhizium anisopliae* acting as an entomopathogenic fungus in order to control the pest and has been experimented within the laboratory as well as in field trials. Furthermore, there is no yet any report on the larvicidal effect of *M. anisopliae* blastospore against *Ae. albopictus* been studied in Malaysia.

3.0 Research questions

3.1 What is the (LC_{50} and LC_{90}) of *M. anisopliae* blastospore against *Ae. Albopictus*?

3.2 What is the lethal time of different concentrations of *M. anisopliae* blastospore against *Ae. Albopictus*?

4.0 Research hypothesis

4.1 *Metarhizium anisopliae* blastospore is highly efficacious against *Ae. albopictus larvae.*

4.2 High concentration of *M. anisopliae* blastospore has shorter death time on the *Ae. albopictus* larvae.

5.0 Objectives

5.1 To determine the LC50 and LC90 of *M. anisopliae* blastopore against *Ae. albopictus*.

4.2 To determine the LC50 and LC90 of *M. anisopliae* blastospore against *Ae. albopictus.*

6.0 Literature review

6.1 *Aedes albopictus*

Mosquitoes are common, flying blood-sucking arthropods that live in most parts of the world. Mosquitoes already become a significant pest in many countries because they are closely associated with human and other vertebrate animals (CDC, 2020). Mosquitoes are an example of arthropod vectors that transmit disease through their blood-feeding abilities. This will occur when the arthropod directly injects the pathogen either bacteria, parasite, or virus into the bloodstreams of the organism they are feeding on. The pathogen also gets into the host's body when the arthropod vector chews through the host skin. *Aedes albopictus* can be characterized by a striking white and black pattern. *Aedes albopictus* is an arthropod that belongs to the *Culicidae* Family and Genus *Aedes* (ECDC, 2019). *Aedes albopictus* is an epidemiologically important vector especially for the transmission of many viral pathogens, including dengue fever, Chikungunya fever and yellow fever (Motoki et al., 2019). The life cycle of *Ae. albopictus* takes about 7-10 days for an egg to develop into an adult mosquito. Female mosquitoes will lay eggs near a stagnant pool or in containers that hold water and the eggs will hatch when covered with water. The eggs will hatch into larvae within 24 hours (CDC, 2020).

6.2 Mosquito Controlling Methods

Dengue fever is a mosquito-borne viral infection caused by *Ae. albopictus* (Motoki et. al., 2019). There are no specific treatments for dengue fever and because of that, the main control that can be done is by destroying the mosquito's habitat and controlling their population. Mosquito control has mainly been dependent on chemical pesticides. Chemical control involves the application of products designed to kill mosquitoes, either in the larval stage through physical damage or hormonal disruption, or also in the adult stage through nervous system disruption, but due to overuse of the chemical pesticides, there is an increase of resistance in mosquito populations against the insecticides itself (Accoti, 2021). To overcome this problem, many studies have been done including the recent study regarding entomopathogens fungi which is *M. anisopliae* against *Ae. Albopictus* larvae (Malassigné et al., 2020). However, there is still a lot of research need to be done to confirm the studies are successful.

6.3 *Metarhizium anisopliae*

Metarhizium anisopliae has been used as a biological control agent for insect pests and has been experimented within the laboratory as well as in field trials (Alkhaibari et al., 2018). *Metarhizium anisopliae* is an anamorphic fungus under the phylum of Ascomycota, the order of Hypocreales and Family of *Claviciptaceae* (Alkhaibari et al., 2018). For its appearance, originally, white fungal hyphae will cleave from the cuticle of the arthropods and as it matures, it will change into olive-green color characteristic. As the arthropod lives in the open environment, the conidia will stick to the exoskeleton of the

arthropods and germinate. Next, a germ tube will grow which has a flattened and thickened tip known as appressorium end. A penetration peg will then grow under the appressorium, pierces the exoskeleton, and enter hemocoel (Malassigné et al., 2020). Finally, a single cell known as blastospore will bud off from the penetration part, circulate into the arthropod's hemocoel, multiplying, consuming, and decreasing the host nutrient intake, ending up killing off the host.

6.4 Pathogenicity of *M. anisopliae blastospore*

Metarhizium anisopliae can be divided into two types of spores which are conidia and blastospores. Both of these spores have their own virulence factor that will act specifically towards the mosquitoes' larvae. They adhere to the surface of terrestrial arthropod hosts and penetrate the cuticle using a combination of enzymes which are hydrolytic enzymes such as protease, lipase, chitinases, and as well as mechanical force (Alkhaibari et al., 2018). Blastospores differ from conidia in several ways, especially their characteristic which is blastospore presence with a thin-walled, pleomorphic, hydrophilic spores produced relatively inexpensively due to short fermentation times within 2–3 days in liquid media, while conidia are uniform-shaped, hydrophobic spores produced within 12–20 days on solid substrates such as rice (Malassigné et al., 2020). The reasons why exactly blastospores are more aggressive is still unclear but blastospores are normally considered more virulent than conidia as they form germ tubes and penetrate the host integument more rapidly than conidia (Alkhaibari et al., 2018). However, the

action of the virulence factor of blastospores is different depending on mosquitoes' larvae species.

7.0 Materials and methods

7.1 Fungal Culture

Pure primary culture of *M. anisopliae* was obtained from Universiti Putra Malaysia (UPM). Then, the Sabouraud Dextrose Broth (SDB) was prepared by suspending 21 grams of the formulated ingredients into 700 ml distilled water that later can be filled into 7 conical flasks. Next, the broth inside the conical flask is sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes. After being autoclaved, the broth was cooled down before subculturing the fungus in Sabouraud Dextrose Broth (SDB). Finally, the subculture was incubated aerobically at 25-30 °C for five days on the shaker.

7.2 Maintenance of the mosquitoes's larvae

The egg of *Ae. albopictus* was received from Universiti Sains Malaysia (USM). The eggs were hatched in the shallow's trays filled with the unchlorinated water that was prepared 24 hours before and mixed with the fish food (tetra pro) and managed at a room temperature of 27± 2 °C, in 16L: 8D photoperiod. Bacterial growth is the main factor that triggers the eggs to hatch by de-oxygenate the water and inducing the first instar to hatch within 12 hours of dehydration. The feed was given several at an interval of 1 to 2 days. Daily observation was done due to the homogenous population of late 3^{rd} or early 4^{th} instars can be obtained within two to three days later.

7.3 Blastospore Suspension

The blastospore suspension has been prepared by separating out the blastospore in the Sabouraud Dextrose Broth using sterile lens film paper. After that, the blastospore suspension concentration was observed and counted using a Neubauer hemocytometer. The hemocytometer was filled with the blastospore suspension and then left for 30 seconds to allow the spores to settle in the chamber. Next, the hemocytometer was viewed under a compound microscope until visualization of the four large corner squares. Each corner square was made up of 16 small squares. The suspension concentration was calculated by multiplying the average spore counted in the large corner square with its volume. The volume for each large corner square is 0.1µl which is equal to 10^4/ml. Therefore, the blastospore suspension concentration was determined by multiplying the average number of spores by 10^4/ ml.

7.4 Larvicidal Bioassays

The mosquitoes' larvae were exposed to a different range of concentrations and control to see their reactions as a final result. For the bioassay's procedure, the batches of 25 third instar mosquitoes' larvae were transferred from the tray into small disposable cups using a dropper. The blastospore suspension with concentration 10^5 blastospore/ml was prepared and then followed by other subsequent concentrations (10^3 and 10^1 blastospore/ml). Two more replicates were set up for each concentration and an equal number of controls were also set up simultaneously by using non chlorinated tap water. Each test was conducted three times on different days. All the disposable cups filled with

blastospore suspension and mosquitoes' larvae were held at 25–28 °C with 12 hours light followed by 12 hours in dark.

7.5 Observation of Larval Motility

After 24 hours of exposure, larval mortality was recorded. Moribund larvae are counted and added as dead larvae for mortality percentage calculation. Moribund larvae are those that are incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. The larvae were confirmed dead by observation under the dissecting microscope and being probed with a needle in the siphon or the cervical region to ensure there is no longer any movement. The results were recorded in the provided table based on the concentration of the blastospore (Table 8.1). Observation and record of the larvae mortality was conducted for 7 days.

8.0 Results

On the first day of observations, the larvae within all concentration did not show or appeared to have any reduction in their larvae activity but during the second day, the larvae started to appear to be less motile and inactive in the highest concentration of *M. anisopliae* suspension which is 10^5. The larvae appeared to be more less motile and inactive in the 10^5 concentrations compared to other concentrations which are 10^1 and 10^3. These larvae show reduction in ability to rise into the water surface and less in their mobility. The larvae were reduced in their diving when the larvae were probed with the dropper. By the fourth day of observations, the larvae within the highest concentration (10^5) have a total reduction in their movement and no capability to rise to the water surface while for 10^3 and 10^1 reach their total reduction on the seventh day.

The percentage of larvae mortality results were recorded in Table 8.1. Based on the table shown, for the first day of observations, there was no presence of moribund and dead larvae in any concentrations. But on the second day of observation, there started to be larval mortality within concentrations of 10^5, 10^1 and 10^3 at 61.68%, 5% and 2.50% respectively. The highest larval mortality percentage was observed on the fifth day of observation at 100% under concentrations 10^5. The different mortality results between the concentrations, revealed that 10^5 has caused the highest mortality based on table 8.1.

Table 8.1: The percentage (%) of Ae. albopictus Larvae mortality.

Days/ Blastospore concentrations	The percentage (%) of larvae death for each concentration		
	1x 10^5	**1x 10^3**	**1x 10^1**
Day 1	0	0	0
Day 2	61.68	5.00	2.50
Day 3	65.00	13.33	6.68
Day 4	66.68	19.18	9.18
Day 5	100	21.68	13.33
Day 6	100	28.33	17.50
Day 7	100	41.68	20.00

Table 8.2: The cumulative percentage (%) of moribund and dead larvae in different concentrations of treatment.

Concentrations of treatment	Cumulative Percentage (%) of moribund and dead larvae	P-value
10^1	20.00	P >0.05
10^3	41.68	
10^5	100	

Probit Analysis was conducted to determine the (LC_{50} and LC_{90}), and (LT_{50} and LT_{90}) for the blastospore suspension 5.6 x 10^4 conidia/ml and 7.5 x 10^6 respectively. Meanwhile, for the median lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}), concentrations of 10^5 was the shortest lethal time taken to cause total larvae mortality compared to the others concentrations (10^1 and 10^3) which is at 4.32 days and 18.8 days respectively. Meanwhile, the lowest concentration 10^1 has the longest

lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) at 23.53 days and 132 days based on table 8.3.

Besides that, A one-way ANOVA was conducted to determine the association of larval motility within different concentrations. The results show that there is no significant difference between concentrations of the treatments and larval mortality as the P >0.05.

Table 8.3: Lethal time 50 (LT_{50}) and lethal time 90 (LT_{90}) of Aedes albopictus against Metarhizium anisopliae blastospore.

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})	23.53	15.71	4.32
lethal time 90 (LT_{90})	132.0	73.39	18.8

After the larvae died, the larvae then incubated in a sealed petri dish at room temperature for final observations of mycelia growth on the larvae cadaver under the dissecting microscope. There are two stages of *M. anisopliae* growth on the larvae cadaver, which is the growth of white mycelia within the first stage and growth of green discoloration of mycelia that indicates matured mycelia in the second stages. White mycelia growth can be observed on the larvae cadaver on the day-2 post mortality in concentrations 10^1. While for the full growth of green mycelium can be seen formed on the larvae cadaver on a day-2 post mortality in the highest concentration 10^5 and day-3 post mortality in the concentration 10^3. The result was recorded in table 8.3.

Table 8.4: Mycelium growth on the Ae. albopictus larvae cadaver post-mortality

Post-mortality Days	*Aedes albopictus* larvae cadaver		
	10^1	10^3	10^5
Day 2			
Day 3			
Day 4			

9.0 Discussion

In this study, it is shown that the highest concentration of *M. anisopliae blastospore* which is 10^5 can affect and reduce the larvae activity mostly by increasing the adhering of blastospore on the larvae body especially the cuticle part. The blastospore adhere to the surface of terrestrial arthropod hosts and penetrate the cuticle using a combination of enzymes which are hydrolytic enzymes such as protease, lipase, chitinases, and as well as mechanical force (Alkhaibari et al., 2018). This condition later would lead to formation of germ tubes and penetrate the host integument more rapidly and leading to the decrease in larval behaviour activity. Therefore, this will be resulting in the decreasing of larvae activity such diving characteristics and no capability to rise to the water surface.

Secondly, the LC_{50} and LC_{90} for the blastospore suspension are $5.6x10^4$ conidia/ml and $7.5x10^6$ conidia/ml respectively. The results cannot be compared with any other results from the case study because based on our knowledge there is no any record have been found and this is the first research that studied about the efficacy of *M. anisopliae blastospore* towards the *Ae. albopictus* larvae. Therefore, the results from this study might be a reference for future research. But based on overall results, it still can be concluded that *M. anisopliae blastospore* are effective to cause 50% mortality towards the *Ae. albopictus* larvae with $5.6x10^4$ conidia/ml concentration that have been achieved in this study. On the other hands, several factors such as geographical distribution, climate and other parameters will also influence the outcome result for the study.

Next, based on this study also, it shown that the highest concentration of *M. anisopliae blastospore* which is 10^5, took a shorter time to cause mortality towards

the larvae rather than the lower concentration that took more longer time. The highest concentration of *M. anisopliae blastospore* which is 10^5 took only four days' time to cause 100% larvae mortality rather than other concentrations which is 10^3 and 10^1. The time taken for death is usually dependent on the infection dose and the virulence factor which in this case, *M. anisopliae blastospore* will adhere to the surface of the host cuticle and follow by the colonization of hemocoel. Besides that, blastospores appeared to be more dependent on entry using mechanical force and specific enzymes that later would affect the *Ae. albopictus* larvae (Malassigné et al., 2020). Therefore, high concentration of *M. anisopliae blastospore* indicated high virulency that can cause higher numbers of larvae mortality. However, based on the case study, the LT_{50} that was achieved is within 24 hours period but in this case, the larvae required more than 24 hours to show the effectiveness. The LT_{50} and LT_{90} for this study is about 4 days and 19 days respectively. But in this case, the LT_{90} is not properly valid as the *Ae. albopictus* life cycle only took seven days to complete from eggs until become an adult mosquito.

Furthermore, the ANOVA result shows that there is no significant association between the concentrations of blastospore suspension (10^5, 10^3 and 10^1) with the larval mortality rate with P-value of >0.05. This shows that the larvicidal effect of the blastospore suspension is highly not associated with the blastospore concentrations in order to produce a high amount of mortality of *Ae. albopictus* larvae. But the comparison between the concentration still can be made by differentiate the lethal time for each concentration where the LT_{50} and LT_{90} for 10^5 concentrations took about 4 days and 19 days while for 10^3 concentration it takes about 16 days and 73 days and lastly for the 10^1 concentration that took the longest

time to cause mortality towards the *Ae. albopictus* larvae for about 24 days and 132 days respectively,

Lastly, the growth and development of the mycelia on the larvae cadaver after death was confirm that the larvae were dead due to the exposure of *Metarhizium anisopliae referring to* Hänel, 1982. The dead larvae of *Ae. albopictus* were incubated at room temperature for four days in order to observe the formation of mycelia, because the study has shown mycelia would form within 96-120 hours. The formation of white mycelia occurs 4 days post-mortality, followed by formation of green mycelia at the 5 days post mortality. 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 within 96-120 hours (Hänel, 1982). The rate for mycelium formation was also related with the concentration factor, where the dead larvae in the higher concentration suspension have visible mycelium formation in a relatively short period of time rather than in the lower concentration suspension. Blastospore were visible in all concentrations starting on day-4 post mortality and then transformed into mycelium on the larvae cadaver on day-5 post mortality. (T. Jaronski, 2014).

10.0 Conclusion

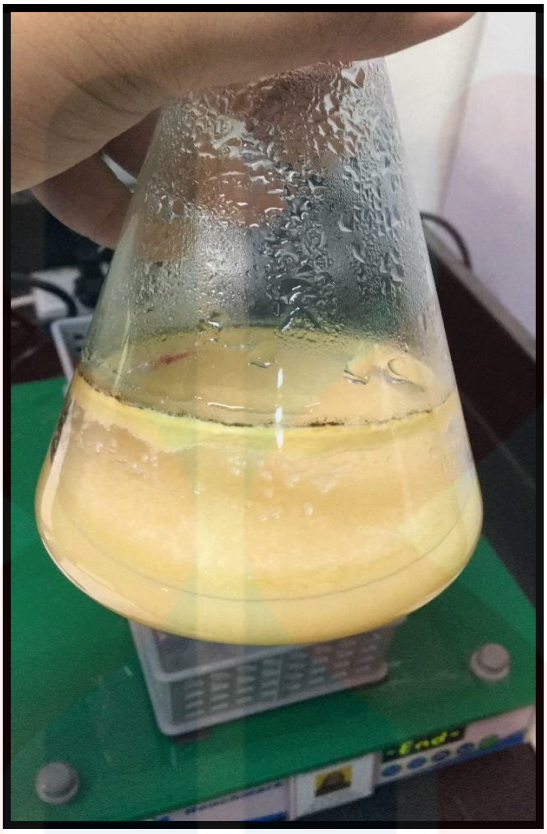
In conclusion, the overall Larvicidal Effects of *M. anisopliae Blastospore* Against *Ae. albopictus* was relatively high in highest concentration of fungal suspension which is 10^5 supported by LC50 and LC90 a value of $5.6x10^4$ conidia/ml and $7.5x10^6$

conidia/ml respectively that appeared to be virulence in inducing the larval motility of *Ae. albopictus.*

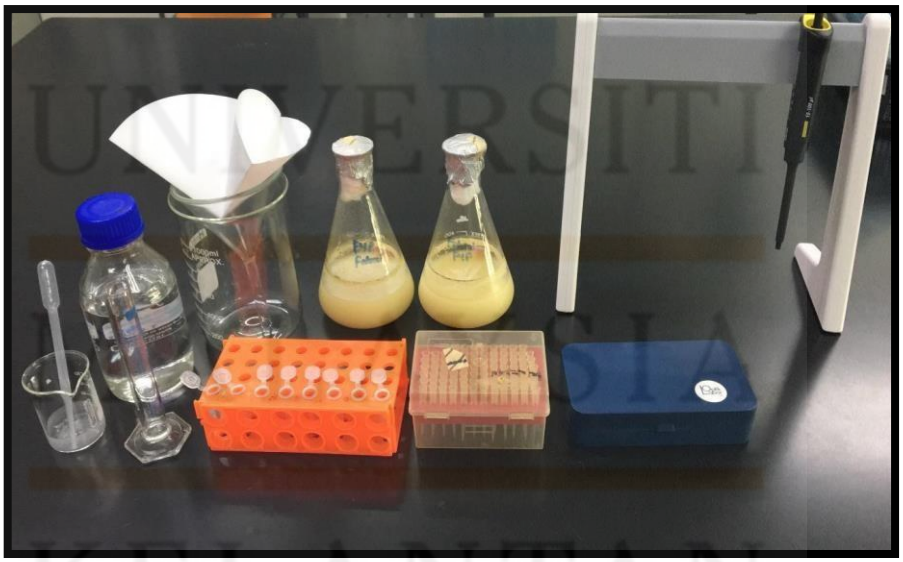
11.0 Recommendations and future work

The effectiveness of *M. anisopliae* as a biological control against *Ae. Albopictus* was highly recommended. Hence further research about its efficacy and toxicity with more specific methods were suggested. It is recommended that a further study of the larvicidal effects of the fungus towards different species of *Aedes* mosquito should be done because it will help in determining the potency of the fungus in inhibiting the larvae growth development. Other than that, a study on the pathogenicity of the fungus on the different larva stage and adult of *Ae. albopictus* was also highly suggested, in order to produce a complete observation of the entomopathogenicity of *M. anisopliae* on all life stages of *Ae. Albopictus* by sending the sample of dissection of dead larvae to the histopathology lab. Lastly, it is also recommended to conduct the study in the proper place such as a specific room with a proper temperature so there will be no other factors that can disturb the outcome findings.

Appendix A

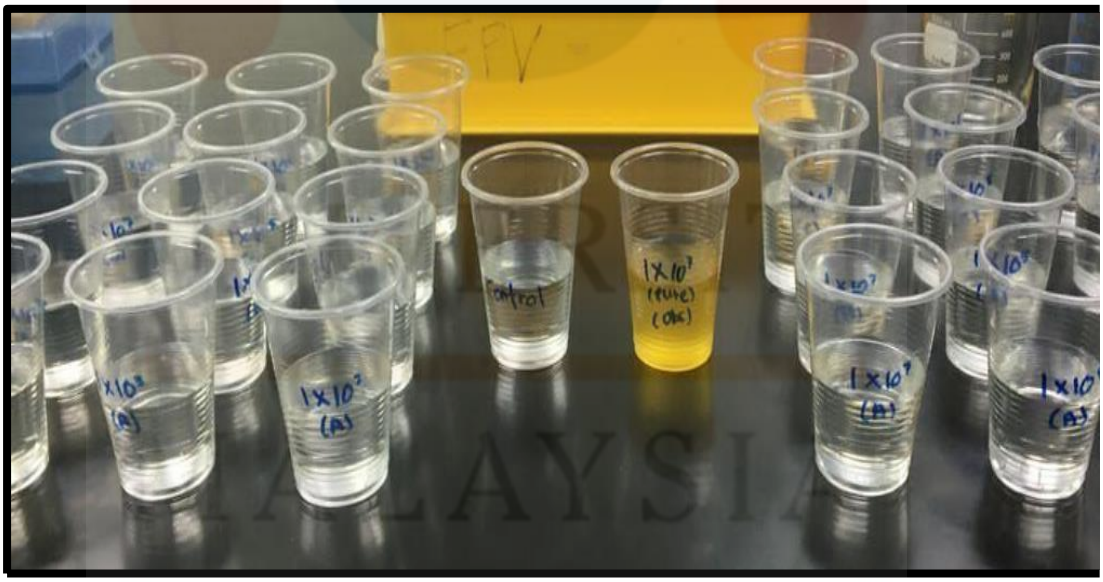


Appendix A.1: Metarhizium anisopliae blastospore suspension in Sabouraud Dextrose Broth



Appendix A.2: Materials and tools for bioassays

Appendix A.3: Aedes albopictus 3rd and 4th instar larvae



Appendix A.4: Larvicidal bioassay of Metarhizium anisopliae blastospore

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