THE INTEGRATED VECTOR MANAGEMENT OF THE SYNERGISM FORMULATION BETWEEN *Ipomoea cairica* PLANT EXTRACT AND *Metarhizium anisopliae* META-G4 ON *Aedes* MOSQUITOES

Wan Fatma Zuharah[*1], Rohaiyu Binti Md Rodzay[1], Wahizatul Afzan Azmi[3], Hideyuki Nagao[2]

[1]Medical Entomology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

[3]School of Marine and Environmental Sciences, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia.

*Corresponding email: *wfatma@usm.my*

ABSTRACT

The integrated vector management (IVM) is a new concept to optimize the use of resources to maximize the efficacy and sustainability of the vector control. Considering the excellent performances of *Metarhizium anisopliae* fungi and *Ipomoea cairica* leaf extract against *Aedes* larvae, here we investigated the possibility of integrated synergism effects of these two natural agents into one formulation. The larvicidal effectiveness and the persistence of this formulation were tested against primary and secondary vectors of dengue fever, *Aedes aegypti* and *Ae. albopictus.* The study revealed the compatibility of *I. cairica* leaf extracts and *M. anisopliae* fungi was at the maximum level of 450 ppm and 6×10^6 conidia/mL, respectively, in which were able to endure the spore production of *M. anisopliae* by estimating using curves estimation regression models. The synergistic effects of these two integrated agents had shown high susceptibility and caused faster larval mortality for *Aedes albopictus* at 100% within 6 days of the treatment periods and 15 days for *Ae. aegypti*. The effectiveness of these integrated agents was reduced to less than 50% after day 21. The current finding shows that the combination of *M. anisopliae* with *I. cairica* can produce strong synergistic interaction justified the possibilities of combining these two agents into one formulation and can prolong the effectiveness in controlling the dengue vectors. In facts, the effectiveness of this formulation towards both *Aedes* larvae has approved the potential to be used in IVM to curb dengue diseases.

Keywords: *Aedes*, Integrated Vector Management, *Ipomoea cairica*, *Metarhizium anisopliae*, Synergism

ABSTRAK

Pengurusan perosak bersepadu (IVM) merupakan konsep baru bagi mengoptimum penggunaan sumber untuk memaksimumkan keberkesan dan kelestarian kawalan vektor. Dengan mengambilkira prestasi cemerlang oleh kulat *Metarhizium anisopli*ae dan ekstrak daun *Ipomeoa cairica* terhadap terhadap larva *Aedes*, kami mengkaji kebarangkalian kesan

sinergisme bersepadu di antara kedua agen semulajadi di dalam satu formulasi. Keberkesanan larvisidal dan kerintangan formulasi ini diuji terhadap vektor utama dan kedua demam denggi, *Aedes albopictus* dan *Ae. aegypti*. Kajian menunjukkan keserasian ekstrak daun *I. cairica* dan kulat *M. anisopliae* adalah pada tahap maksima 450 ppm dan 6×10^6 conidia/mL, yang mana mampu untuk berlansung terus dengan produksi spora *M. anisopliae* menggunakan anggaran model regrasi. Kesan sinergisme oleh kedua agen integrasi menunjukkan kerentanan yang tinggi dan menyebabkan kematian larva yang lebih cepat terhadap *Aedes albopictus* pada tahap 100% dalam tempoh 6 hari tempoh rawatan dan 15 hari untuk *Ae. aegypti.* Keberkesanan integrasi agen menurun sehigga 50% selepas hari ke-21. Hasil kajian semasa menunjukkan kombinasi *M. anisopliae* dengan *I. cairica* boleh menghasilkan interaksi sinergatik yang kuat memberikan justifikasi kebarangkalian untuk menggabungkan kedua agen di dalam satu formulasi dan boleh memanjangkan keberkesanan dalam pengawalan vektor deggi. Secara fakta, keberkesanan formulasi ini terhadap kedua larva *Aedes* membuktikan potensi untuk digunakan dalam IVM untuk mencegah penyakit denggi.

Kata kunci: *Aedes*, Pengurusan Vektor Bersepadu, *Ipomoea cairica*, *Metarhizium anisopliae*, Sinergisme

INTRODUCTION

Aedes aegypti and *A. albopictus* are the incriminated dengue vectors prefer to breed in natural and man-made containers (Madzlan et al. 2017) that responsible for the great strides of dengue transmissions in Malaysia (Chen et al. 2005). In Malaysia, the main dengue vector control methods rely extensively on chemical insecticides to control the risk of dengue reinfestation and reduce the target *Aedes* population involved in the dengue transmission area (Lee 2005). Although the use of insecticide is useful to control the larval and adult populations of *Aedes* mosquitoes, yet the effect of extensive usages will affect non-target insects, accumulation of toxic in the environment and rapid development of insecticide resistance (Frentiu et al. 2014; Paula et al. 2011). The concept of integrated vector management is a combination of more than two management approaches with the primary goals to improve the efficacy, cost-effectiveness and sustainability of vector control program. One of the leading candidates for mosquito control is entomopathogenic fungus, *Metarhizium anisopliae* (Frentiu et al. 2014). However, the efficiency of this fungus is limited due to its slow killing activities, low persistence in environments, spore viability and number of spores available to fulfil the needed for effective control (Kamareddine et al. 2013). Given these confounding factors, the efficacy of *M. anisopliae* could be enhanced by combining with other natural agents through an integrated action known as synergism. Synergism action will increase the potency of each component within the mixture given a combined effect of the new formulated mixture greater than individual effects (Bhan et al. 2013). Here, we designed the integrated agents between *M. anisopliae* fungi and *I. cairica* plant extract to increase the effectiveness and to prolong their residual. The application of larvicides from plant extracts derivative also had been proven as one of the potential mosquito control agents (Ghosh et al. 2012). *Ipomoea cairica*, morning glory, a widely growing shrub in Malaysia recently has received considerable attention as a new potential bioactive agent in the vector control management (AhbiRami et al. 2014; Thiagaletchumi et al. 2014). Here we studied for the first time the combination of *I. cairica* leaf extracts with *M. anisopliae* as a newly formulated bio-larvicides. The combined action of these both agents is expected to facilitate the larvicidal action against *Aedes* larvae by the rapid larvicidal action from *I. cairica* leaf

extracts, and the survival larvae will be killed by the slower larvicidal action from *M. anisopliae* for the prolonged action.

MATERIALS AND METHODS

Rearing of Mosquitoes

The Vector Control Research Unit (VCRU) laboratory strains of *Ae. albopictus* and *Ae. aegypti* used in this study had been established and maintained in the Entomology Laboratory, Vector Control Research Unit, Universiti Sains Malaysia at the temperature of 28±2ºC and 70-85% relative humidity. The mosquitoes were reared until reached 3rd and early 4th instar to be used in the larvicidal assay experiments.

Plant Extract

The plant samples of *I. cairica* was collected around Universiti Sains Malaysia, segregated and dried under room temperature for about two weeks. The dried leaves were mechanically ground into fine powders using a commercial blender (Panasonic: MX-899TM) to provide a greater surface area during the extraction process. Leaves powders at 40gm were inserted into the paper thimble and extracted using Soxhlet apparatus. Acetone was chosen as the solvent with the boiling point at 50.5°C and extracted for three cycles. The crude yield then further dried up using rotary evaporator (Brand Buchi) and kept in the fridge at 4°C until further used. The serial concentration of *I. cairica* for larval bioassay testing was prepared ranging from 30- 1000 ppm diluted in distilled water.

Fungi Culture

Entomopathogenic fungi, *M. anisopliae* META-G4 strain was isolated from agricultural soil in Felda Tenang, Setiu, Terengganu. Prior to the experiment, the fungi colonies were sub-cultured on PDA at 28℃ for 14 days before being used in the experiment. The total conidial spores counted using haemocytometer and were multiplied by 10,000 (10^4) indicated the current final conidial suspension concentrations.

Minimum Inhabitation Concentration (MIC) Test

A series of *I. cairica* extracts were prepared to test the minimum concentration that effects the *M. anisopliae* fungi growth. For the bioassay, 500µL aliquots of extracts ranging between 30-1000ppm were inoculated into a semi-solidified PDA in petri dishes and allowed to solidify inside the laminar flow. The initial standard fungal at 1x 10^6 conidia/ml were spread on the PDA with *I. cairica* extracts and kept in the dark storage at 28°C for the germination process. The growth of mycelia mat was checked on day 5, and the spores were calculated using haemocytometer. The curves estimation regression model was plotted afterwards.

Synergism Effects

Twenty mosquito larvae of the 3rd and early 4th instar was prepared in paper cups sized 6cm × 10.5cm × 8.5cm with 200ml of seasoned water. Seasoned water is the water that was left for more than 24 hours to reduce the chlorine content. A mixture of 450ppm *I. cairica* plant extracts and *M. anisopliae* fungal suspensions at 1x 10^6 conidia/ml was prepared and added into the test cups. The test was carried out under the laboratory conditions at 28ºC, 75% RH humidity and 12L: 12D. 24h mortality was recorded, and the residual effect was checked until the efficacy decreased to 50%. Both bioassays for *Ae. albopictus* and *Ae. aegypti* larvae were done separately.

Statistical Analysis

The highest compatibility values from the integrated synergism effect of *I. cairica* leaf extracts incorporated with *M. anisopliae* culture medium was determined by using a curve estimation regression model in SPSS 20.0. Whereas, the effectiveness of these combination agents on the larval mortality for each species and residual time was calculated using two-way ANOVA in SPSS 20.0. The data were log-transformed prior to analysis.

RESULTS

The best integrated synergism effect from the curve estimation regression between *M. anisopliae* fungi and *I. cairica* plant extract was found at the 6 x 10^6 conidia and 450 ppm; respectively. At this combination, the spore fungal growth was found growing at the maximum rate as shown in Figure 1.

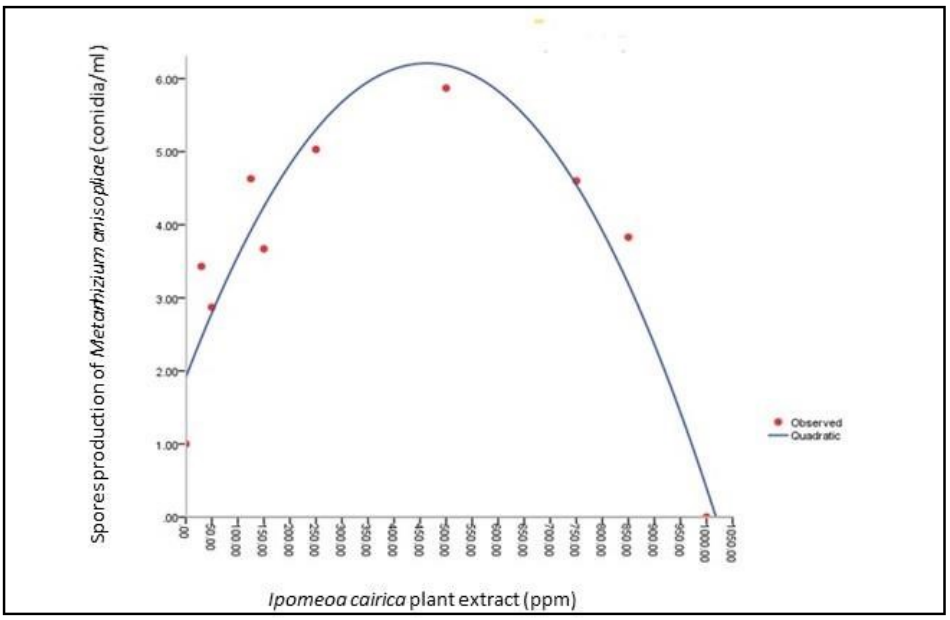

Figure 1. The curves estimation regression between *Metarhizium anisopliae* and *Ipomoea cairica*

In the bioassay testing, *Ae. albopictus* was more susceptible to the integrated agents as compared to *Ae. aegypti*. In which, the mortality for *Ae. albopictus* reached 100% on the day 6 and for *Ae. aegypti* was on day 15 (Figure 2). However, no significant effect was found between species (MS=4.898, F=0.773, p= 0.718; Table 1), but the treatment period showed significant differences (P<0.05). The maximum synergism effects on 100% mortality for both *Aedes* species were on day 15 and the effectiveness reduced below 50% after 21 days post-treatment. No mortality on both *Aedes* species was found in the control treatment.

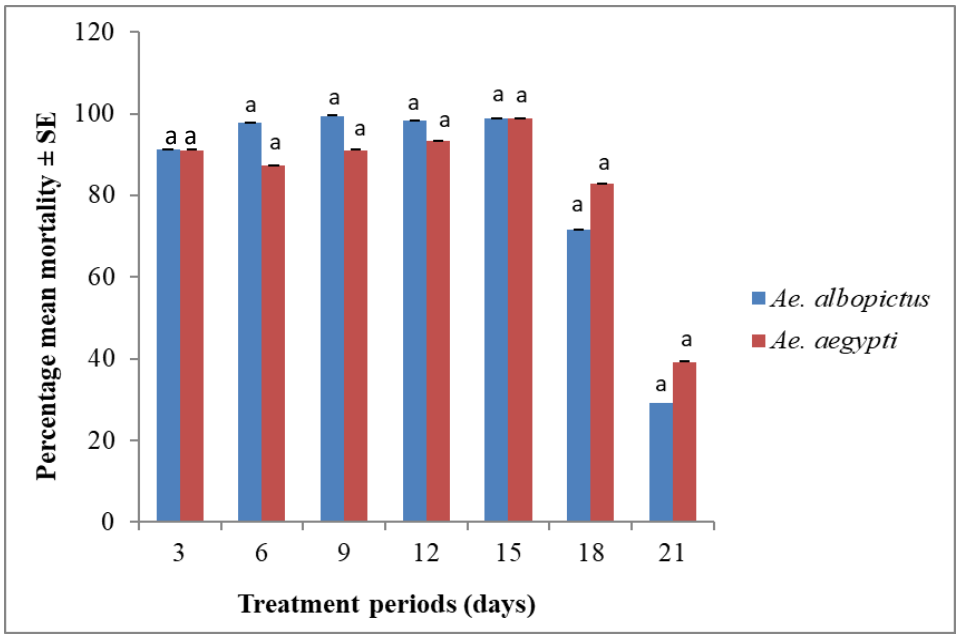

Figure 2. The residual effects on the larval mortality of *Aedes albopictus* and *Ae. aegypti* after the exposure to the integrated mixture of *Metarhizium anisopliae* fungi and *Ipomoea cairica* plant extract. The letter represents the comparison between mosquito species for each treatment periods.

Table 1. Two-way ANOVA results of the integrated effects of *Metarhizium anisopliae* fungi and *Ipomoea cairica* extract on the mortality on post-treatment days of *Aedes albopictus* and *Ae. aegypti*.

Source	***df***	**Mean square**	***F*-value**	***P*-value**
Aedes species	1	4.898	0.773	0.718
Treatment periods	17	29.103	4.595	**0.000***
Aedes x treatment periods	17	11.133	1.758	0.143

DISCUSSION

Our study demonstrated the effectiveness of integrated synergism effects between a combinations of *I. cairica* plant extracts *M. anisopliae* fungus treatments by causing the mortality towards 3rd instar larvae of *Ae. albopictus* and *Ae. aegypti.* A similar result has shown by Gomes et al. (2015) for the combination of neem and fungus, which significantly reduced larvae survivability than those caused by single treatment alone. Recently, several attempts have been made to increase the effectiveness of this fungus by combining fungal conidia with plant extracts derivatives (Gomes et al. 2015; Islam et al. 2010; Radha et al. 2014). In order to determine the optimal combination of entomopathogenic fungi with plant extracts, detailed compatibility studies are required (Sahayaraj et al. 2011). This is because some of the plant extracts showed significant toxicity effects that may inhibit the germination of the fungal spores (Islam et al. 2011). Here, we have found out the best compatibility level is at 450 ppm for *I. cairica* and 6×10^6 conidia/mL for *M. anisopliae*. In which, this concentration of *I. cairica* has not caused inhibition of the spore production of *M. anisopliae*.

The rate of fungal infection highly depended on the spore production since the first steps of the infection process is through germination spore in the treated samples (Oliveria et al. 2003).

In our larvicidal bioassay, it was found that both *Ae. albopictus* and *Ae. aegypti* larvae were highly susceptible to this formulation. The highly effective mechanisms for this formulation presumably resulted from the integrated, synergistic interaction of plant extract-fungus treatments. As synergism will affect the larval humoral defenses system with two different modes of action (Bhan et al. 2013), this presumably explained the high mortality rate of *Aedes* larvae observed in this study. In facts, this synergism also not only increase the mortality rate, but it also produces a rapid killing effect on the treated insects. As observed in this study, the rapid killing effect by the *I. cairica* leaf extracts overcoming the slow virulence of *M. anisopliae* towards the treated larvae. The active secondary metabolites lining within the plant extract such as alkaloids were known to inhibit the acetylcholinesterase (Ache) action that can cause the accumulation of acetylcholine at the synaptic pathway (Rattan 2010). This inhibition potentially caused toxin and eventually lead to rapid mortality effect on the larvae. Whereas, for *M. anisopliae*, the mortality effect entails by colonization and invasion of fungal spore which limits the ability for faster infection rate (Shah and Pell 2003). Together, by combining these two different modes of action, we modified the fungal-plant effectiveness by increasing the virulence and speed of kill of the products, adding the weights to the possibility of using this formulation for field implementation.

Here, we can conclude that choosing a complimentary compound for the combined mixture clearly play a major part to produce the integrated synergism effect. In facts, this new formulation also can facilitate the killing effect of larval and increase the fungal persistence. Completing our research aim, we verified the possibility of combining *I. cairica* leaf extracts with *M. anisopliae* as a new formulation that might be useful for the IVM for *Aedes* larvae.

ACKNOWLEDGEMENTS

The authors are grateful to the staff of Vector Control Research Unit and School of Biological Sciences, USM for field assistance. This project was supported by Research University Grant, Universiti Sains Malaysia (1001/ PBIOLOGI/8011066).

REFERENCES

AhbiRami, R., Zuharah, W.F., Thiagaletchumi, M., Subramaniam, S. & Sundarasekar, J. 2014. Larvicidal efficacy of different plant parts of railway creeper, *Ipomoea cairica* extract against dengue vector mosquitoes, *Aedes albopictus* (Diptera: Culicidae) and *Aedes aegypti* (Diptera: Culicidae). *Journal of Insect Science* 14(1):180.

Bhan, S., Shrankhla, L.M. & Srivastava, C.N. 2013. Larvicidal toxicity of temephos and entomopathogenic fungus, *Aspergillus flavus* and their synergistic activity against malaria vector, *Anopheles stephensi*. *Journal of Entomology and Zoology Studies* 1(6):55-60.

Bhan, S., Shrankhla, L.M. & Srivastava, C.N. 2013. Larvicidal toxicity of temephos and entomopathogenic fungus, *Aspergillus flavus* and their synergistic activity against malaria vector, *Anopheles stephensi*. *Journal of Entomology and Zoology Studies* 1(6):55-60.

Chen, C.D., Lee, H.L., Stella-Wong, S.P., Lau, K.W. & Sofian-Azirun, M. 2009. Container survey of mosquito breeding sites in a university campus in Kuala Lumpur, Malaysia. *Dengue Bulletin* 33: 187-193.

Frentiu, F.D. Walker, T. & O' Neill, S.L. 2014. Biological control of dengue and *wolbachia*-based strategies. In. Gubler, D.J (Ed.). *Dengue and Dengue Hemorrhagic Fever,* 2nd ed., pp. 537-547. Oxford, UK.: CABI.

Ghosh, A., Chowdhury, N. & Chandra, G. 2012. Plant extracts as potential mosquito larvicides. *Indian Journal Medical Research* 135(5): 581-598.

Gomes, S.A., Paula, A.R., Ribeiro, A., Moraes, C.O., Santos, J.W., Silva, C.P. & Samuels, R.I. 2015. Neem oil increases the efficiency of the entomopathogenic fungus *Metarhizium anisopliae* for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. *Parasites & Vectors* 8(1):669.

Islam, M.T., Olleka, A. & Ren, S. 2010. Influence of neem on susceptibility of *Beauveria bassiana* and investigation of their combined efficacy against sweetpotato whitefly, *Bemisia tabaci* on eggplant. *Pesticide Biochemistry and Physiology* 98(1):45-49.

Kamareddine, L., Fan, Y., Osta, M.A. & Keyhani, N.O. 2013. Expression of Trypsin Modulating Oostatic Factor (TMOF) in an entomopathogenic fungus increases its virulence towards *Anopheles gambiae* and reduces fecundity in the target mosquito. *Parasites & Vectors* 6(1):22.

Lee, H.L. 2005. Germ warfare against mosquitoes. What now. *Proceedings of the Fifth International Conference on Urban Pests*, pp. 9-18.

Madzlan, F., Dom, N.C., Zakaria, N., Hasnan, S.N.A., Tiong, C.S. & Camalxaman, S.N. 2017. Profiling of dengue vectors breeding habitat at urban residential areas in Shah Alam Malaysia. *Serangga* 22(2): 185-216.

Oliveria, G.N., Neves, P.M.O.J. & Kawazoe, L.S. 2003. Compatibility between the entomopathogenic fungus *Beauveria bassiana* and insecticides used in coffee plantations. *Scientia Agricola* 60(4):663-667.

Paula, A.R., Carolino, A.T., Paula, C.O. & Samuels, R.I. 2011. The combination of the entomopathogenic fungus *Metarhizium anisopliae* with the insecticide imidacloprid increases virulence against the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Parasites & Vectors* 4(1):8.

Radha, R., Murugan, K., Wei, H., Amerasan, D., Chen, P.M.F., Kovendan, K., Nataraj, T., Nareshkumar, A., Hwang, J.S. & Govindarajan, M. 2004. Insecticidal activity of essential oils and entomopathogenic fungi against Cowpea Bruchid, *Callosobruchus maculatus* (F.) (Insecta: Coleoptera: bruchidae). *International Journal of Current Innovation Research* 1:1.

Rattan, R.S. 2010. Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Protection* 29(9): 913-920.

Sahayaraj, K., Namasivayam, S.K.R. & Rathi, J.M. 2011. Compatibility of entomopathogenic fungi with extracts of plants and commercial botanicals. *African Journal of Biotechnology* 10(6):933-938.

Shah, P.A., & Pell, J.K. 2003. Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* 61(5):413-423.

Thiagaletchumi, M., Zuharah, W.F., Rami, R. A., Fadzly, N., Dieng, H., Ahmad, A.H. & AbuBakar, S. 2014. Assessment of residual bio-efficacy and persistence of *Ipomoea cairica* plant extract against *Culex quinquefasciatus* Say mosquito. *Tropical Biomedicine* 31(3):466-476.