Biocontrol Potentiality Of Entomopathogenic Fungi Against Larvae Of Dengue Fever Vector, *Aedes aegypti* (Diptera: Culicidae)

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Abstract: Dengue is an acute mosquito-borne viral infection with global health concern and is transmitted by several species of mosquito within the genus *Aedes*, including *Aedes aegypti*. *Aedes aegypti* is the primary vector of Dengue fever. It imposes a significant socioeconomic and disease burden on many tropical and subtropical regions of the world. The growing insecticide resistance in the primary mosquito vector, *Aedes aegypti*, limits the effectiveness of vector control, therefore exploration of alternative tools are urgently needed. Among the alternative approaches the use of biopesticides comprising entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* able to reduce the mosquito vector longevity which will help in decreasing the disease transmission. Two isolates of entomopathogenic fungi *B. bassiana* and *M. anisopliae* isolated from soil samples has demonstrated its efficacy against mosquito species *Aedes aegypti* in the laboratory condition. The virulence of *B. bassiana* and *M. anisopliae* was tested against 2nd instar larvae of *Aedes aegypti* using five concentrations 10^4, 10^5, 10^6, 10^7 and 10^8 conidia/ml respectively. The larval mortality was observed for a period of 10 days. Results showed that the larval mortality of mosquito larvae treated with *B. bassiana* in different fungal concentrations varied from 12-60% mortality. However, mortality treated with different conidial concentrations of *M. anisopliae* showed better results and recorded mortality rate in the range of 17-100 %. It was also observed that the larval mortality rate increases with increasing concentration of conidia in both the entomopathogenic fungal isolates. The exposure of 10^6 conidia/ml and 10^8 conidia/ml concentration the *M. anisopliae* showed highest (100%) larval mortality within 6 to 8 days respectively, where as in case of *B. bassiana* isolate a maximum of 60% mortality was observed with conidial concentration 10^6 conidia/ml after 8 days of treatment in laboratory condition. The present findings indicate that both entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* has the potential to be used as biocontrol agent for Dengue mosquito vector *Aedes aegypti*.
Keywords: Dengue, *Aedes aegypti,* Entomopathogenic fungi, *Beauveria bassiana, Metarhizium anisopliae*

Introduction

Mosquitoes borne diseases including malaria, filariasis, yellow fever, dengue fever and Japanese encephalitis, contribute significantly to poverty and social debility in tropical countries (Rajkumar and Jebanesan, 2005; Madkour *et al*., 2014). The *Aedes* mosquito is considered to be one of the world's most important mosquito vector species not only because of its susceptibility to these disease agents, but also because it often feeds on more than one individual during a single gonotrophic cycle (Platt *et al.,* 1997). Mosquitoes are still the serious global threat to public health transmitting several diseases including Dengue which has been emerging as a major cause of concern in the world. *Aedes* mosquitos are predominantly active during daylight hours which pose difficulties in controlling the vector.

The dengue disease is now endemic in more than 100 countries in Africa, Americas, Eastern Mediterranean, South-east Asia and Western Pacific regions. Southeast Asia

and the Western Pacific are the most seriously affected (WHO, 2010). WHO estimates that over 2.5 billion people (40% of the global population) are live in dengue prone areas where as 100 million cases of dengue and Dengue Heamorrhagic Fever (DHF) are reported in the world every year. Amongst the 50 million hospitalizations cases for dengue hemorrhagic fever, 90% are children (WHO, 2010) and 2.5% of those affected are dying.

Aedes aegypti is a highly anthropophilic and is an efficient epidemic vector of several human diseases such as dengue fever, Chikungunya, and yellow fever (Harrington *et al.,* 2001).

Aedes mosquito thrives in urban and semi-urban areas, ovipositing eggs in a wide range of man-made containers and cryptic microhabitats in and around houses (Hawley, 1988; Chadee, 2004). All of these making the rapid establishment of the *Aedes* spp. as a potential vector of Dengue, chikungunia and other vector borne diseases (Hawley, 1988; WHO, 2013).

Aedes aegypti, the primary vector for dengue viruses (DENV) that cause dengue and dengue hemorrhagic and yellow fevers, is found mainly in the tropics and subtropics (Langat *et al*., 2012). Dengue viruses (DV) belong to family *Flaviviridae* and there are four serotypes of the virus referred to as DV-1, DV-2, DV-3 and DV-4. All the four serotypes are transmitted mainly by *Aedes aegypti* mosquito and also by *Ae. albopictus* (Gupta, 2012). The expansion of dengue is expected to increase due to factors such as the modern dynamics of climate change, globalization, travel, trade, socioeconomic settlement and also viral evolution. There is no effective vaccine or specific antiviral therapy currently exists to address the growing threat of dengue. Thus, the only way of significantly lowering the incidence of this disease is through mosquito control (Murray *et al*., 2013).

The most convenient method for mosquito control especially Dengue vectors lies in eradication of breeding sites and application of environment friendly larvicides (Certin *et al*., 2004). However, the common approach for the control of mosquito vectors and reducing the transmission of human pathogens is mainly based the chemical insecticides (Paul *et al*., 2006). The use of chemical insecticides is still the most important element in mosquito control programmes (Alves *et al*., 2002). The major constrains associated with the chemical insecticides includes, gaining vector resistance in the mosquito population, environmental pollution, human health and economic costs have led to the search for alternative control agents. In recent years, efforts have been made all over the world on the search for natural, eco-friendly resources derived from plants and microorganisms as an alternative to conventional chemical insecticides for insect-control (Quesada-Moraga *et al*., 2006; Madkour *et al*., 2014). Many biological control agents have been evaluated to determine their efficacy for control of mosquito vectors at larval stages of mosquitoes and reported successful including *Bacillus thuringiensis israelensis* variety and *Bacillus sphericus* (Fillinger *et al.,* 2003).

Among the promising biological control agents entomopathogenic fungi plays an important role for *Aedes* control as potent bio-control agent (Boucias and Pendland, 1998). Entomopathogenic Ascoomycetes notably *M. anisopliae* and *B. bassiana* are among the most commonly encountered insect pathogens (Goettel and Inglis, 1997) and are in use to manage various arthropod pest species (Zimmermann, 1993; Khetan, 2001). The use of entomopathogenic fungi against a range of mosquito larvae has been the subject of various studies (Clark *et al*., 1988; Alves *et al*., 2002). Effectiveness of entomopathogenic fungi against different mosquito larvae under laboratory conditions had been reported earlier (Murry *et al.,* 2013; Benserradj and Mihoubi, 2014; Madkour *et al*., 2014) and also observed higher variability of effectiveness at killing mosquito larvae in the field condition (Goettel, 1987). Besides, insect resistance, their impact on the environment has necessitated the development of other means of vector control strategies. The most recent environment friendly strategy for the *Aedes* control is the biological control.

Study has been conducted to evaluate the pathogenicity of the two naturally occurring soil-born entomopathogenic fungus *Beauveria bassiania* and *Metarrhizium anisopliae* on the 2nd instar *Aedes aegypti* larvae under laboratory conditions.

Materials and methods

Laboratory rearing of *Aedes aegypti*

Aedes aegypti eggs were collected from Indian Council of Medical Research; as a generous gift from Dr. Anil Prakash, Deputy Director, ICMR, Dibrugarh, Assam, India. Mosquitoes were reared by establishing a colony in the Department of Zoology, Gauhati University following standard method (Silva *et al*., 1998). Larvae were fed with a mixture of dog biscuit (Pedegree) and yeast powder (3:1). Adults were maintained at 27 (± 2) °C, 85 (± 10%) RH in a 12:12 h L: D photoperiod. Blood meal was provided by placing a rabbit in the rearing cage three times in a week. Males were provided with cotton pads soaked in 5% sucrose solution ad libitum. Oviposited eggs were collected in a filter paper (Whatman No.1) wrapped inside round a beaker half filled with water. After harvesting, the eggs were dried in room temperature and eclosion was stimulated by total immersion of the filter paper in deionized water.

Isolation and culture of fungi

Fungal strains *Beauveria bassiana* and *Metarhizium anisopliae* were isolated from the soil samples collected from different locations of western part of Guwahati city, Assam, India through serial dilution methods described by Nakayama, (1981). Soil samples were ground to fine powder and mixed with sterilized water in a ration of 1 g soil : 100 ml of sterilized water. The solution was then diluted serially up to five times and inoculated onto sterile culture plates containing 0.5 g K_2HPO_4, 0.5 g peptone, 0.5 g $MgSO_4$, 10 g dextrose, 0.5 g yeast extract, 0.05 g rosebengal, 0.03 g streptomycin sulphate. The plates were incubated at 25°C for a period of 7-10 days. Pure culture were established according to single spore method by inoculation of the primary culture on PDA medium and incubated for a period of 5 to 7 days at 25°C. Isolates were maintained in culture on potato dextrose agar (PDA) slants and stored at 4°C. Continuous cultures were maintained on slopes, with sub-cultures grown for 14 days at 25°C and stored at 4°C.

Laboratory bioassay

Newly sporulated conidia were harvested by scraping the surface of the culture gently with inoculation needle and were suspended in distilled water containing 0.01% tween80. The mixture was stirred with a magnetic stirrer for 10 minutes. Fine mesh sieve was used to remove the hyphal debris by filtering the mixture. The conidial final suspension was maintained at different conidial concentrations by using haemocytometer. Conidial suspension of the concentration at $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia/ml was prepared by diluting with water and mixed with 0.01% tween80. Fungal isolates were tested for conidial viability one day before each bioassay as described by Goettel and Inglis (1997) and only isolates with not less than 80% viability were used in bioassays.

Conidia of *B. bassiana* and *M. anisopliae* were tested against *Aedes* mosquitoe larvae by adding fungal suspension to plastic cups containing 50 ml of distilled water with 20 larvae of the 2nd instar. Each cup was inoculated with 1ml of fungal suspensions (10^8, 10^7, 10^6, 10^6, 10^4 conidia/ml). Control treatments were carried out by addition of 10 ml of distilled water. Larval mortality was evaluated on a daily basis for 10 days. Three replications were used in each set to compare the result and statistically viable. Mortality percentages were recorded daily and inference were made to know the effective concentration of the strain applied against the 2nd instar larvae of *Aedes aegypti*.

Results

The result of the present findings shows the pathogenic activity of both the fungal strains *B. bassiania* and *M. anisopliae* on the 2nd instar larvae of *Ae. aegypti*. The larval mortality of Aedes mosquito larvae treated with *Beauveria bassiana* in different fungal concentrations varied from 12-60% mortality (Table 1), whereas treatment with different conidial concentrations of *Metarhizium anisopliae* showed better response with respect to immobilization and mortality and recorded mortality rate in the range of 17 to 100% (Table 2).

Among the various treatments the exposure of 10^6 conidia/ml and 10^8 conidia/ml concentration of the *Metarhizium anisopliae* showed the highest larval mortality (100%) within 6 to 8 days respectively, where as in case of

Fig. 1 Photomicrographs showing (a). Aedes larvae after 3 days of treatment. (b). Mycelial growth after 5 days of treatment and (c). Larral body parts degradation after 8 days of treatment of *Beauveria bassiana.*

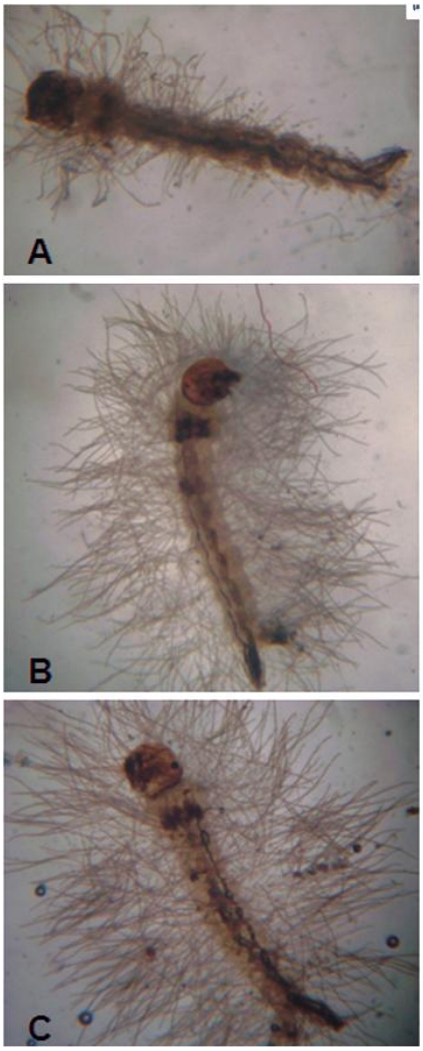

Fig.2. *Aedes aegypti* 2nd instar larvae after exposure to *Metarhizium anisopliae*.

Beauveria bassiana isolate a maximum of 60% mortality was recorded with conidial concentration 10^6 conidia/ml after 8 days of treatment in laboratory condition.

It was also observed that the mortality rate of larvae increased with the increasing concentration of conidia and duration of treatment in both the fungal isolates. In our study, after exposure of *B. bassiana* maximum 60% of mortality was recorded from day 8 to day 10 in the 10^6 conidia/ml concentration and the lowest 40% was recorded at the concentration of 10^7 conidia/ml from day 6 to day 10. More than 50% mortality was recorded from day 4 to day 10 and recorded the maximum in the higher 10^8 conidia/ml. showing the morphological deformities of the larvae (Fig.1)

In case of *M. anisopliae* 100% mortality were recorded from day 6 to day 10 in the conidial suspension containing 10^6 to 10^8 conidia/ml respectively (Fig. 2). Similarly, 80% mortality of the treated larvae was recorded in day 5 after being exposure of 10^6 conidia/ml concentration. It was observed that more than 60% in day 4 and more than 40% in day 3 were recorded in the same concentration.

Table 1. Mortality (%) of *Aedes aegypti* 2nd instar larvae after exposure to *Beauveria bassiana*. (values are mean ± SE)

	Control	10^4 conidia/ml	10^5 conidia/ml	10^6 conidia/ml	10^7 conidia/ml	10^8 conidia/ml
1st Day	0	0	0	0	0	0
2nd Day	0	0	13.33±0.66	0	16.67±0.33	26.67±0.33
3rd Day	0	0	13.33±0.66	0	16.67±0.33	43.33±0.33
4th Day	0	13.33±0.33	13.33±0.66	0	16.67±0.33	56.67±0.33
5th Day	0	13.33±0.33	13.33±0.66	26.67±0.33	16.67±0.33	56.67±0.33
6th Day	0	13.33±0.33	13.33±0.66	26.67±0.33	40±1.00	56.67±0.33
7th Day	0	13.33±0.33	13.33±0.66	26.67±0.33	40±1.00	56.67±0.33
8th Day	0	13.33±0.33	13.33±0.66	60±0.57	40±1.00	56.67±0.33
9th Day	3.33±0.33	13.33±0.33	13.33±0.66	60±0.57	40±1.00	56.67±0.33
10th Day	3.33±0.33	13.33±0.33	13.33±0.66	60±0.57	40±1.00	56.67±0.33

Table: 2. Mortality (%) of *Aedes aegypti* 2nd instar larvae after exposure to *Metarhizium anisopliae*. (values are mean ± SE)

	Control	10^4 conidia/ml	10^5 conidia/ml	10^6 conidia/ml	10^7 conidia/ml	10^8 conidia/ml
1st Day	0	0	0	13.33±0.66	0	0
2nd Day	0	0	0	13.33±0.66	0	26.66±0.88
3rd Day	3.33±0.33	13.33±0.33	26.67±0.88	53.33±0.88	16.67±0.33	26.66±0.88
4th Day	3.33±0.33	13.33±0.33	26.67±0.88	73.33±1.20	16.67±0.33	43.33±0.88
5th Day	3.33±0.33	13.33±0.33	26.67±0.88	83.33±0.33	16.67±0.33	63.33±0.88
6th Day	3.33±0.33	23.33±0.88	26.67±0.88	100±00	16.67±0.33	76.67±1.20
7th Day	3.33±0.33	23.33±0.88	26.67±0.88	100±00	56.67±1.66	86.67±0.88
8th Day	3.33±0.33	23.33±0.88	26.67±0.88	100±00	56.67±1.66	100±00
9th Day	3.33±0.33	23.33±0.88	40±0.57	100±00	56.67±1.66	100±00
10th Day	3.33±0.33	23.33±0.88	40±0.57	100±00	56.67±1.66	100±00

Discussion

Pathogenic activity of the fungal isolates in reducing the mosquito population has been in use as a tool in the present-day biological control programme (Ernst-Jan Scholte *et al.*, 2004). In this study two natively isolated fungal isolates *M. anisopliae* and *B. bassiania* were applied to evaluate the entomopathogenic activity against the immature stage of the primary Dengue vector *Aedes aegypti* as these stages are reported to be most perfect stage for infection by the bio-control agents (Yap, 1985; Conti *et al.*, 2010). The result represented the pathogenic activity of both the fungal isolates exhibiting 12-60% mortality in case of *B. bassiana and* 17 to 100 % mortality in case of *M. anisopliae*, where as the control exhibits only 3% mortality in case of both the isolates with 10 days exposure period. Similar type of result was also reported with rapid killing of mosquito larvae after application of fungal conidia of hypomycetic fungi (Mvoutoulou *et al.*, 1992; Costa *et al.,* 1998 and Moraes *et al.*, 2001).

The study confirmed the pathogenicity of *B. bassiana* isolates and reported maximum mortality up to 60% with conidial concentration of 10^6conidia/ml after 8 days of treatment. Moreover, more than 50% from day 4 to day 10 in the concentration of 10^8conidia/ml and the lowest 40% from day 6 to day 10 in the 10^7 conidia/ml concentration were observed respectively, but 100% mortality could not be achieved by application of any one of the five conidial concentrations of *B. bassiana*. This can be explained as the

conidia of *B. bassiana* are hydrophobic, thus floating on the water surface and contact mosquito larvae at the tip of the siphon resulting slow progression of pathogenicity. Similarly, Clark *et al.,* (1968) confirmed that *B. bassiana* conidia are effective in killing mosquito larvae only when it is applied as a conidial dust to the water surface.

Our study also revealed increasing level of mortality with respect to the increasing level of conidial concentration and duration of treatment in case of *M. anisopliae* and recorded maximum 100% mortality from day 6 to day 10 in the 10^6 to 10^8 conidia/ml concentration respectively. Similarly 80% mortality in day 5 at 10^6 conidia/ml concentration and 40% in day 3 were recorded in the same concentration. This finding was consistent with the study made by Roberts 1970 and observed the potentiality of *M. anisopliae* conidia in a wide range of species of mosquito including *Anopheles stephensi*, *Anopheles quadrimaculatus*, *Aedes aegypti*, *Ochlerotatus atropalpus*, *Ochlerotatus taeniorhynchus*, *Culex pipiens*, *Culex restuans* and *Culex salinarius*. *M. anisopliae* as a potent microbial mosquito control agent has also been demonstrated by Roberts (1970) due to its high germinating power and persistence in the environment as well as its effect on *Culex pipiens, Aedes aegypti* larvae causing 50% mortality of the above larvae treated with 1 mg dry conidia per 16 cm^2 (Daoust and Roberts, 1982).

In conclusion it can be referred that *B. bassiana* and *M. anisopliae* are potential mosquito controlling agents. Researchers observed effects of these fungi on larvae of several species of mosquitoes. Furthermore, it showed that *M. anisopliae* fungus can successfully infect and kill larvae of *Aedes* species. These isolates may also be used in mosquito control programs in North East India alone or perhaps in combination with other bio-control agents for proper control of *Aedes aegypti*.

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References

Alves, S.B., Alves, L.F.A., Lopes, R.B., Pereira, R.M. and Vieira, S.A. 2002. Potential of some *Metarhizium anisopliae* isolates for control of *Culex quinquefasciatus* (Diptera, Culicidae). J Appl Ent. 126: 504–509.

Benserradj, O. and Mihoubi, I. 2014. Larvicidal activity of entomopathogenic fungi *Metarhizium anisopliae* against mosquito larvae in Algeria. Int J Curr Microbiol App Sci. 3(1): 54-62.

Boucias, D.G. and Pendland, J.C. 1998. Principles of Insect Pathology. Kluwer Academic Publishers, Boston, Massachusetts.

Cetin, H., Erler, F. and Yanikoglu, A. 2004. Larvicidal activity of a botanical natural product, AkseBio2, against *Culex pipiens*, Fitoterapia. 75 (7-8): 724-8.

Chadee, D.D. 2004. Key premises, a guide to *Aedes aegypti* (Diptera: Culicidae) surveillance and control, Bull. Entomol. Res. 94. 201–207.

Clark, T.B., Kellen, W., Fukuda, T. and Lindegren, J.E. 1968. Field and laboratory studies on the pathogenicity of the fungus *Beauvaria bassiana* to three genera of mosquitoes. Journal of Invertebrate Pathology. 11: 1-7.

Conti, B., Canale, A., Bertoli, A., Gozzini, F. and Pistelli, L. 2010. Essential oil composition and larvicidal activity of six Mediterranean aromatic plants against the mosquito *Aedes albopictus* (Diptera: Culicidae). Parasitol Res. 107: 1455–1461.

Costa, G.L. and Oliveira, P.C. 1998. *Penicillium* species in mosquitoes from two Brazilian regions. Journal Basic Microbiology. 38: 343-347.

Daoust, R.A., Ward, M.G. and Roberts, D.W. 1982. Effect of formulation on the virulence of *Metarhizium anisopliae* conidia against mosquito larvae. Journal of Invertebrate Pathology. 40: 228- 236.

Ernst-Jan Scholte, Knols, B.G.J., Samson, R.A. and Takken, W. 2004. Entomopathogenic fungi for mosquito control: A review. Journal of Insect Science. 19 (4): 24

Fillinger, U., Knols, B.G.J. and Becker, N. 2003. Efficacy and efficiency of new *Bacillus thuringienesis var.israelensis* and *B. sphaericus* formulations against the malarial vector *Anopheles gambiae* in Western Kenya. Tropic Med Int Healt. 8: 37- 48.

Goettel, M.S. 1987. Preliminary field trials with the entomopathogenic Hyphomycete *Tolypocladium cylindrosporum* in Central Alberta. J American Mosquito Control Association. 3: 239- 245.

Goettel, M.S. and G.D. Inglis. 1997. Fungi: Hyphomycetes. In: Manual of Techniques in Insect Pathology, Lacey, L.A. (Ed.). Academic Press, New York, San Diego, USA. 213-250.

Gupta, N. 2012. Dengue in India. Indian J Med Res. 136: 373-390.

Harrington, L.C., Edman, J.D. and Scott, T.W. 2001. Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood. J Med Entomol. 38(3): 411–422.

Hawley, W.A. 1988. The biology of *Aedes albopictus*. J Am Mosq Control Assoc Suppl. 1:1–39.

Khetan, S.L. 2001. Microbial pest control. Marcel Dekker, Inc, New York. 211-256, ISBN 9780824704452.

Murray, N.E.A., Quam, M.B. and Wilder-Smith, A. 2013. Epidemiology of dengue: past, present and future prospects. Clinical Epidemiology. 5. 299–309.

Madkour, M.H., Zaitoun, A.A. and Singer, F.A. 2014. Innovative biocontrolling method of dengue fever vector, *Aedes aegypti* (Diptera: Culicidae). Journal of Agricultural Science. 6 (9): 208-213.

Moraes, A., Costa, G., Barcellos, M., Oliveira R. and Oliveira, P. 2001. The entomopathogenic potential of *Aspergillus* spp. in mosquitoes vectors of tropical diseases. Journal of Basic Microbiology. 41: 45-49

Paul, A., Harrington, L.C and Scott, J.G. 2006. Evaluation of novel insecticides for control of dengue vector *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. 43: 55.

Platt, K.B., Linthicum, K.J., Myint, K.S., Innis, B.L., Lerdthusnee, K. and Vaughn, D.W. 1997. Impact of dengue virus infection on feeding behavior of *Aedes aegypti*. Am J Trop Med Hyg. *57*: 119-125.

Quesada-Moraga, E., Carrasco-Díaz, J.A. and Santiago-Álvarez, C. 2006. Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep: Noctuidae). Journal of Applied Entomology. 130: 442–452.

Rajkumar and Jebanesan. 2005. Oviposition deterrent and skin repellent activities of *Solanum trilobatum* leaf extract against the malarial vector *Anopheles stephensi*. Journal of Insect Science. 15(5): 3.

Roberts, D.W. 1967. Some effects of *Metarhizium anisopliae* and its toxins on mosquito larvae. In: Insect pathology and microbial control. Ed. Van der Laan, Amsterdam. 243-246.

Silva, H.H.G., Silva, I.G. and Lira, K.S. 1998. Metodologia de criação, manutenção de adultos e estocagem de ovos de *Aedes aegypti* (Linnaeus, 1762) em laboratório. Rev Patol Trop. *27*: 53-63.

WHO. 2010. Dengue and severe dengue, Fact sheet No.117, Updated February 2015.

WHO. 2010. Regional office for Southeast Asia. World Health Organization. Regional Office for Southeast Asia Dengue case fatality rate. Available from: http:// www.searo.who.int/LinkFiles/Dengue_85-06.pdf.

WHO. 2013. World malaria report, Geneva. Pp: 284.

Yap, H.H. 1985. Biological control of mosquitoes, especially malaria vectors *Anopheles* species. Southeast Asian J Trop Med Public Health. 16: 163–172.

Zimmermann, G. 1993. The entomopathogenic fungus *Metarhizium anisopliae* and its potencial as a biocontrol agent. Pesticide Science. 37. 375-379.