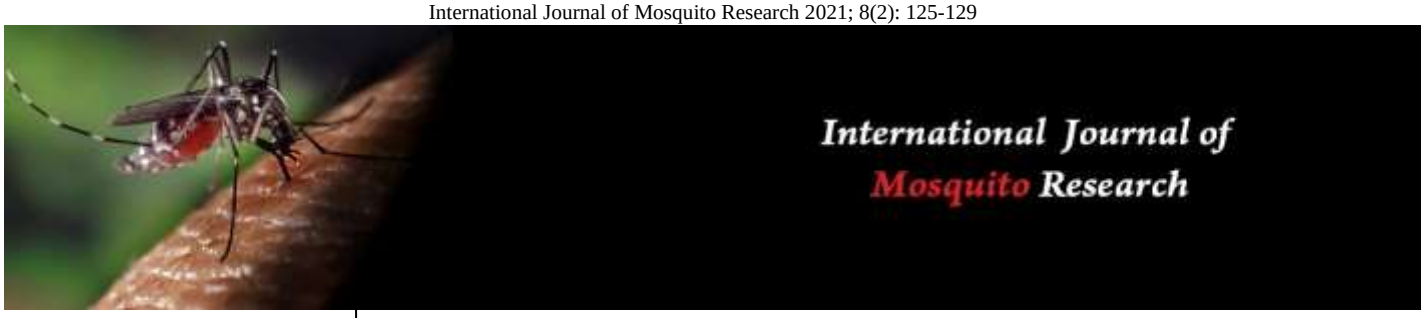

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2021; 8(2): 125-129

www.dipterajournal.com
Received: 12-01-2021
Accepted: 25-02-2021

Panukorn Boonsit
Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand

Dr. Itsanun Wiwatanaratanabutr
Director of Center of Excellence in Applied Biosciences and Associate Professor at Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Chalongkrung Rd., Ladkrabang, Bangkok 10520, Thailand

Corresponding Author:
Dr. Itsanun Wiwatanaratanabutr
Director of Center of Excellence in Applied Biosciences and Associate Professor at Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Chalongkrung Rd., Ladkrabang, Bangkok 10520, Thailand

First report on the integration of copepods and *Wolbachia* to control *Aedes* larvae in Thailand

Panukorn Boonsit and Dr. Itsanun Wiwatanaratanabutr

Abstract
Wolbachia is a group of *Rickettsia*-like, intracellular, maternally inherited bacterial endosymbionts that infect a diverse range of arthropods and cause reproductive changes in their hosts. *Mesocyclops thermocyclopoides* is a species of copepod crustacean that naturally infected with *Wolbachia*. While its role in some hosts has been studied extensively, its incidence among copepod species is still largely unknown. Here, we present the first report on the study of the integration between copepods and *Wolbachia* (*Wolbachia*-infected copepods) compared with *Wolbachia*-uninfected copepods (Tetracyclin-treated) to determine the predatory efficacy for controlling on the *Aedes* larvae under laboratory conditions. The main objective was to examine if *Wolbachia*-infected *M. thermocyclopoides* had a better efficiency for *Aedes* control than *Wolbachia*-uninfected ones. Based on the findings, the copepods with *Wolbachia* infection had a higher predatory efficiency than uninfected ones. Therefore, they could be recommended as potential candidates for biocontrol agents of *Aedes* mosquitoes in the future.

Keywords: Infection, mosquito, copepod, *Wolbachia*, *Aedes*, larvae

1. Introduction
Wolbachia, the most common and widespread intracellular bacterium on Earth, is a genus of maternally inherited bacteria that infect a diverse range of arthropods. Around *20-76%* of all insect species are estimated to be infected by *Wolbachia* bacteria [1,2]. Some *Wolbachia* strains can sustain themselves in their hosts and decrease adult lifespan. *Wolbachia* can be involved in the manipulation of their host's reproductive system, resulting in different abnormal reproductive phenotypes including cytoplasmic incompatibility, male killing, feminization, and parthenogenesis [2,3,4]. These phenotypes promote the distribution of *Wolbachia* in host populations by increasing the numbers of infected females. The best known of these traits are Cytoplasmic Incompatibility (CI) which its intensity is affected by many factors such as temperature and rearing density [5,6,7] and Feminization.
The CI occurs when infected males mate with uninfected females or with females that harbor a different *Wolbachia* strain, and results in the death of the fertilized egg [8]. Feminization is a trait first reported in isopod crustaceans that harbor *Wolbachia* [9]. Infected males become morphologically functional females whereas their genetics remain male, causing sex ratio distortion [10]. *Wolbachia* have been studied in many groups of insects such as copepods, mosquitoes, planthoppers, beetles and wasps [3, 7, 11-16]. Sequence analysis of 16S rRNA gene shows that members of the genus *Wolbachia* belongs to a monophyletic clade in the alphaproteobacterial group which is closely related to the *Ehrlichia* assemblage [17].
Several countries are searching for more sustainable and effective methods for controlling mosquito vectors because of the danger and inefficiency of chemical in vector control assays. Biocontrol of mosquitoes using copepods is an effective method of using interactions in nature for vector control with minimum consequences on the environment. Copepods are small crustaceans which feed on a variety of prey including mosquito larvae. Freshwater copepods are one of the important organisms in aquatic ecosystem because they are food for many fish species. The *Mesocyclops* is a genus of copepod crustaceans in the family Cyclopidae that many species of *Mesocyclops* are known to prey on mosquito larvae, it is used as an inexpensive and nontoxic form of mosquito biocontrol. A variety of species of cyclopoid copepods suppress mosquito larvae under experimental conditions and are an important natural control against dengue fever, malaria and other diseases in tropical countries [18]. In 2013,

Wolbachia infections were first reported as a feminizing agent in the copepod, *M. thermocyclopoides* [12]. Experimental transfer of these feminizing *Wolbachia* among copepod hosts revealed the importance of the host on expression of the feminizing trait that make a reproductive advantage to infected hosts which enable *Wolbachia* to spread rapidly through a host population.

Aedes aegypti, the yellow fever mosquito, is a species of mosquito vector which responsible for the transmission of many vector borne diseases such as dengue fever and Zika fever. They are widespread in tropical and subtropical regions, inhabiting urban environments where they have adapted to breed in artificial containers [19]. *Aedes aegypti* is the most efficient transmitting agents of many diseases to human. Only the female bites for blood that needed for the production and maturation of her eggs. Nowadays, almost 2.5 billion people worldwide are at risk from the dengue incidence every year. The World Health Organization reported that South-East Asia region including Thailand remains as a hot spot for dengue, which approximately 75% of the recent global disease burden of dengue [19].

At present, *Wolbachia* is being developed as a new method for biocontrol strategy against many insect pests and disease vectors. The use of a natural *Wolbachia*-infected predator for biocontrol of *Aedes* mosquito would provide a cost-effective and efficient mechanism for controlling of *Aedes* species with the least impacts on the environment. While its role in some hosts has been studied extensively, its incidence among copepod species is still largely unknown. Here, the integration of copepods and *Wolbachia* (*Wolbachia*-infected copepods) to control *Aedes aegypti* larvae in Thailand was first investigated and reported. The objective of this study was to examine if *Wolbachia*-infected *M. thermocyclopoides* had a better efficiency for *Aedes* control than *Wolbachia*-uninfected ones. Our study of the *Wolbachia* infection in copepods would lay the groundwork for further biological investigations of the *Wolbachia* effects on other hosts and has been very helpful in the utilization of *Wolbachia* for vector control.

2. Materials and methods

2.1 Copepod collection and rearing

Copepod specimens were collected from a diverse variety of freshwater habitats ranging from small ponds, ditches, and other standing water sources to large reservoirs from many regions of Thailand between March 2019 and February 2020. The copepods were collected using a 60 µm mesh plankton net at all depths. Collected samples were poured through a sieve with 2 mm mesh to remove any debris and potential copepod predators and transported to the lab in Bangkok within labelled glass containers. All specimens are kept alive for further investigation and identification. The specimens were sorted and morphologically identified to the species level under dissecting and compound microscopes using standard keys [20].

The copepods that identified as *M. thermocyclopoides* were preserved in absolute ethanol and stored at -20 °C until DNA extraction for PCR detection of *Wolbachia*, and some of them, at least one gravid female, were used to establish monocultures in containers with pond water and reared as colonies for further study. The colony was maintained with *Paramecium* culture and wheat grain was supplied as food. Tetracycline 0.25 mg/ml was applied with *M. thermocyclopoides* in a separate colony for 2 generations in order to remove *Wolbachia* from the copepods. The cultures were maintained under a 12:12 (light: dark) cycle at standard conditions (27 °C and 75 % humidity).

2.2 *Aedes aegypti* colonies

Adult mosquitoes were collected from similar or near the locations with copepod collection, and all the collected mosquitoes were transported to the lab in Bangkok. The morphological identification was done based on the keys used in this study [21], the blood fed females of *Ae. aegypti* were identified and reared until oviposition. Eggs laid by these females were used to establish colonies of *Ae. aegypti* for this study. The established colonies were maintained in cages under standard conditions (27 °C and 75 % humidity) with a 12:12 (light:dark) cycle. These eggs laid were transferred from oviposition cups into hatching trays and allowed to be hatched. First instar larvae of *Ae. aegypti* were used for the predation experiment.

2.3 DNA extraction

DNA from the copepods, *M. thermocyclopoides*, was extracted and examined for the presence of *Wolbachia* strains using *wsp* amplification primers [17]. Whole bodies of each copepod were ground in a hand-held polypropylene homogenizer in a 1.5 ml microcentrifuge tube filled with 100 µl of Salt-Tris-EDTA buffer. The homogenate was heated at 95 °C for 10 min and then centrifuged at 1400g for 1 min. One microliter of the supernatant was used to PCR-screen for *Wolbachia*. The extracted DNA was kept at -20 °C for later use. One microliter of the supernatant was used to PCR-screen for *Wolbachia*.

2.4 Polymerase Chain Reaction (PCR) assay

DNA from *Wolbachia*-infected *Ae. albopictus* (Naturally infected with *Wolbachia*) was used as a positive control for general screening for *Wolbachia* in copepods. The contamination was checked by using double-distilled water as a negative control. The 16S rRNA primers [22] which is the RNA component of the 30S small subunit of a prokaryotic ribosome, were used to check the quality of the DNA extraction from copepods. The primers, *wsp*81F and *wsp*691R [17] for the *Wolbachia* outer surface protein were used to screen for the presence of *Wolbachia*. PCR was done on a thermal cycler using 20 µl reaction mixture volumes. Each reaction contained 0.5 µl dNTPs (10 mM each), 2 µl 10X buffer (Promega), 0.5 µl 20 µM forward and reverse primers, 2 µl 25 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Promega). The following temperature profile was used: a cycle of initial denaturation at 95 °C for 3 min, followed by 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 30 cycles, and final extension at 72 °C for 3 min. Ten microliters of each PCR product were run with a 1-kb ladder on a 1% agarose gel to determine size and the presence of amplified product. Specimens yielding a product of the expected size were scored as positive for *Wolbachia*.

2.5 Experiments on larval predation of copepods

Ten adult female copepods both naturally *Wolbachia*-infected and tetracycline-treated (*Wolbachia*-uninfected) *M. thermocyclopoides* were introduced into two different larval rearing trays filled with 500 ml of deionized water and *Ae. aegypti* 1st instar larvae in total of 100 individuals/tray. The larvae remaining in each container and dead larvae were

observed at 4-hour intervals until 24 hours at 27 °C, under lighting conditions. Finally, the numbers of dead larvae were counted using a magnifying glass every 4 hour and calculated mortality rate of *Aedes* larvae. In case that more than 75% of *Aedes* larvae was eaten, new batches of the 1st instar larvae were newly introduced to maintain the 200 of prey density for another interval. The whole experiment was repeated 6 times for each *Wolbachia*-infected / -uninfected copepods to maintain the accuracy of the findings.

To minimize the effects of other factors on the predation behavior of the copepod, *M. thermocyclopoides*, the larval trays were maintained in separate chambers, and lighting systems were used during the 12-hour lighting period. During the whole study period, human movements were limited to enumeration and reintroduction of larvae. The researchers spent a minimum time (less than 3 minutes) to count the surviving larvae (every 4-hour intervals) to ensure minimum disturbance to larval trays. The predation rates of both *Wolbachia*-infected and *Wolbachia*-uninfected *M. thermocyclopoides* were calculated as the deducted number of the remaining mosquito larvae from the earlier surviving *Ae. aegypti* larvae. Predatory efficiencies were calculated following the published formula [23].

2.6 Statistical analysis

Tukey's pair-wise comparison in SPSS program was used for statistical analysis of the predation rates between *Wolbachia*-infected and *Wolbachia*-uninfected copepods. A $P < 0.05$ was considered significant. Predatory efficiencies of copepods were subjected to square-root transformation. The cluster analysis, analysis of similarities and multi-dimensional scaling were used for the comparison of statistical significance in the overall predation of copepods on *Ae. aegypti*.

3. Results and Discussion

The copepods, *M. thermocyclopoides*, from the field collections were identified to the species level. This study was the first report on the comparison of predation efficiency between *Wolbachia*-infected and -uninfected *M. thermocyclopoides* on the mosquito, *Ae. aegypti* larvae. The presence of *Wolbachia* infections in *M. thermocyclopoides* was examined and confirmed by using *wsp* gene primers and the results clearly showed that all collected *M. thermocyclopoides* were infected with *Wolbachia*. The PCR products of *Wolbachia wsp* gene were about 600 bp. The *Wolbachia*-infected copepods, *M. thermocyclopoides* were also treated with the antibiotic, Tetracyclin for two generations to remove *Wolbachia* from the infected copepods. The *Wolbachia*-uninfected *M. thermocyclopoides* were reared and separated to another colony as no *Wolbachia* infection group. In addition, PCR was also performed to confirm the absence of *Wolbachia* in uninfected copepods from the no *Wolbachia* infection colony. The results indicated that infections of *Wolbachia* were not detected from the copepod colony that no *Wolbachia* infection.

Between the studied copepods, *Wolbachia*-infected *M. thermocyclopoides* had a significant higher predation of *Ae. aegypti* (Mean ± Standard Error; 41.97±2.0) than uninfected copepods (Mean ± Standard Error; 35.37±5.4) within 24 hours (Table 1). Results of the Tukey's pair wise comparison revealed that the predation rates of both types of copepods (*Wolbachia*-infected and *Wolbachia*-uninfected) differed significantly from each other ($P<0.05$ at 95% level of confidence) as indicated in Table 1. Overall, the predation rate of *Ae. aegypti* was significantly high, suggesting that *Ae. aegypti* is fit as a dietary item by these copepods.

Table 1: Average number of *Aedes aegypti* larvae consumed by *Wolbachia*-infected and *Wolbachia*-uninfected copepods, *M. thermocyclopoides*, within 24 hours*

Copepods	Mean number of 1st instar larvae consumed by a copepod			
	1st - 2nd Replications	3rd – 4th Replications	5th – 6th Replications	Mean
Wolbachia-infected *M. thermocyclopoides*	42.8±2.2a	39.5±1.8a	43.6±2.1a	41.97±2.0a
Wolbachia-uninfected *M. thermocyclopoides*	33.4±1.6b	35.2±2.3b	37.5±1.5b	35.37±5.4b

*Values are Mean ± SE, range in parenthesis. The letters (a and b) in a column show significant differences ($P < 0.05$) as tested by the Tukey's pair wise comparison at 95% level of significance.

In addition, the predatory efficiencies also differed significantly between the two types of copepods as shown by the statistics tested ($P<0.05$). *Wolbachia*-infected *M. thermocyclopoides* showed the higher predatory efficiency of 4.20±0.5 for *Ae. aegypti* followed by *Wolbachia*-uninfected *M. thermocyclopoides* as shown in Table 2. As indicated by the Turkey's pair-wise comparison, two significantly different types of copepods were identified based on the mean predation rates of *Ae. aegypti* ($P<0.05$) whereas the effects of *Ae. aegypti* vectors on the predatory efficacy of copepods was non-significant ($P>0.05$). Regarding the overall predatory efficiency of copepods against *Ae. aegypti*, two groups of copepods were observed. The distribution of the copepods into two groups (*Wolbachia*-infected and uninfected *M. thermocyclopoides*) was further verified by the Analysis of Similarities at 5% level of significance.

Table 2: Average predatory efficiencies of *Wolbachia*-infected and uninfected copepods on the 1st instar *Aedes aegypti* larvae

Copepods	Predatory Efficiency for *Aedes aegypti* larval consumption
Wolbachia-infected *M. thermocyclopoides*	4.20±0.5a
Wolbachia-uninfected *M. thermocyclopoides*	3.54±0.7b

*Values are Mean ± SE, range in parenthesis. The letters (a and b) in a column show significant differences ($P < 0.05$) as tested by the Tukey's pair wise comparison at 95% level of significance.

This report was the first study of the integration between *Wolbachia* bacteria and the copepod, *M. thermocyclopoides* (*Wolbachia*-infected *M. thermocyclopoides*) compared with *Wolbachia*-uninfected *M. thermocyclopoides* in the predation efficiency of the 1st instar larvae of *Aedes aegypti*. Although many approaches of vector control such as the use of

larvicides, chemical treatment methods and mechanical source reduction, dengue fever remains as the worst problem in Thailand as well as in other countries. Biological control is the method that can be used to overcome the limitations of the above vector control methods which can be incorporated into the integrated vector management for dengue control. So far, no previous study has been reported on this kind of research. Therefore, this is the first report on the use of *Wolbachia*-infected and uninfected *M. thermocyclopoides* in the predation efficiency of the 1st instar larvae of *Aedes aegypti.*

Among the available methods, the use of biological control agents such as copepods to suppress *Aedes aegypti* populations is one of the sustainable approaches. The copepod, *M. thermocyclopoides* has been reported as predators of immature stages of mosquitoes such as *Aedes aegypti*. It is the most efficient predator which is characterized by a high diversity of breeding habitats including lakes, ponds, and small water pools consisting of high microalgal productivity. *Mesocyclops* spp. have a high potential as biological control agents against mosquito vectors including *Aedes* spp. The *M. thermocyclopoides* has been reported as efficient biological control agents of mosquito larvae due to its higher body size than other copepod species which could be a factor that led to the higher predation rates than the other copepods. Our findings are relevant to ongoing efforts to manage mosquito vector control and to understand the efficiency of copepod populations and their *Wolbachia* infections from Thailand. In addition, this certain result invites further thought on and investigation into our understanding of the evolutionary dynamics of *Wolbachia* infections in copepod hosts across ecosystems.

4. Conclusions

This observational study explains the intensive and substantial undertakings required to address this finding with well-designed research studies. The study of integration between *Wolbachia* and the copepod, *M. thermocyclopoides* (*Wolbachia*-infected *M. thermocyclopoides*) compared with *Wolbachia*-uninfected *M. thermocyclopoides* in the predation efficiency of the 1st instar larvae of *Aedes aegypti* would be the fundamental research for further biological investigations of the *Wolbachia* effects on copepods. Therefore, the copepods with *Wolbachia* infection have been very helpful in the application for vector managements and could be recommended as potential candidates for biocontrol agents of *Aedes* mosquitoes in the future.

5. Acknowledgments

The authors thank a group of students from Department of Plant Production Technology for collecting the copepod specimens used in this study. This work was supported by KMITL Research and Innovation Services (KREF046207), King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

6. References

1. Stouthamer R, Breeuwer JAJ, Hurst GDD. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annual Review of Microbiology, 1999;53:71-102.
2. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 2008;6:741-751.
3. Bagheri Z, Talebi AA, Asgari S, Mehrabadi M. *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. Journal of Invertebrate Pathology 2019;163:1-7.
4. Kajtoch L, Kolasa M, Kubisz D, Gutowski JM, Ścibior R, Mazur MA *et al*. Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. *Scientific Reports*. 2019;9:847.
5. Wiwatanaratanabutr S, Kittayapong P. Effects of temephos and temperature on *Wolbachia* load and life history traits of Aedes albopictus. Medical and Veterinary Entomology 2006;20:300-307.
6. Wiwatanaratanabutr I, Kittayapong P. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. Journal of Invertebrate Pathology 2009;102:220-224.
7. Sumida Y, Katsuk M, Okada K, Okayama K, Lewis Z. *Wolbachia* induces costs to life- history and reproductive traits in the moth, *Ephestia kuehniella*. Journal of Stored Products Research, 2017;71:93-98.
8. Braig HR, Zhou W, Dobson SL, O'Neill SL. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. Journal of Bacteriology 1998;180:2373-2378.
9. Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M., Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. B: Biol. Sci 1992;250:91-98.
10. Rigaud T, Moreau J, Juchault P, *Wolbachia* infection in the terrestrial isopod *Oniscus asellus:* sex ratio distortion and effect on fecundity. Heredity 1999;83:469-475.
11. Wiwatanaratanabutr I. Geographic distribution of *Wolbachia* infections in mosquitoes from Thailand. Journal of Invertebrate Pathology 2013a;114:337-340.
12. Wiwatanaratanabutr I, Distribution, diversity and density of wolbachial infections in cladocerans and copepods from Thailand. Journal of Invertebrate Pathology, 2013b;114:341-345.
13. Wiwatanaratanabutr I. *Wolbachia* infection in leafhoppers and planthoppers: Diversity, density and geographic distribution in tropical rice agroecosystems. Journal of Asia-Pacific Entomology 2015;18:277-282.
14. Carjaval TM, Capistrano JDR, Hashimoto K, Go KJD, Cruz MAIJ, Martinez MJLB *et al*. Detection and distribution of *Wolbachia* endobacteria in *Culex quinquefasciatus* populations (Diptera: Culicidae) from Metropolitan Manila, Philippines. Journal of Vector Borne Diseases 2018;55:265-270.
15. Kolasa M, Kubisz D, Mazur MA, Scibior R, Kajtoch L. *Wolbachia* prevalence and diversity in selected riverine predatory beetles (Bembidiini and Paederini). Bulletin of Insectology 2018;71:193-200.
16. Lopez V, Cortesero AM, Poinsot D. Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*). Journal of Invertebrate Pathology 2018;158:24-31.
17. Zhou W, Rousset F, O'Neil S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society of London. Series B, Biological Sciences 1998;265:509-515.

18. Kay BH, Cabral CP, Sleigh AC, Brown MD, Ribeiro ZM, Vasconcelos AW. Laboratory evaluation of Brazillian *Mesocyclops* (Copepoda: Cyclopidae) for mosquito control. *Journal of Medical Entomology*, 1992;6:9-15.
19. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL. The global distribution and burden of dengue. Nature 2013;496(7446), doi: 10.1038/nature12060
20. Mamaril SR, Fernando CH. Freshwater zooplankton of the Philippines (Rotifera, Cladocera, and Copepoda. Advances in Natural and Applied Science 1978;30(4):109-221.
21. Rattanarithikul R, Panthusiri P**.** Illustrated Keys to the Medically Important Mosquitoes of Thailand**.** Wattana Panich, Bangkok 1994**.**
22. O'Neill SL, Giordane R, Colbert AME, Timothy K, Robertson H. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with CI in insects. Proceedings of the National Academy of Sciences of the United States of America 1992;89:2699-2702.
23. Chitra T, Murugan K, Kumar AN, Madhiyazhagan P, Nataraj T, Indumathi D *et al*. Laboratory and field efficacy of Pedalium murex and predatory copepod, *Mesocyclops longisetus* on rural malaria vector, *Anopheles culicifacies*. Asian Pacific Journal of Tropical Diseases 2013;3(2): 111-8.