ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2015; 2 (2): 33-37

Received: 27-03-2015
Accepted: 19-05-2015

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Synergistic larvicidal potential of Temephos and entomopathogenic fungus, *Aspergillus flavus* against filarial vector, *Culex quinquefaciatus* (Say)

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Abstract
In the present study, we tested different combinations of Temephos and *Aspergillus flavus* to observe synergism. Larvicidal activity of Temephos and *A. flavus* was evaluated separately and also together in different ratios, 1:0.5, 1:1, 1:2 and 1:4 against third instar larvae of *Culex quinquefaciatus*. The fungal extract showed LC_{50} 13.616, 14.347, and 10.027 ppm after 24, 48 and 72 hrs, respectively. The LC_{50} values for Temephos were 0.0060, 0.0055 and 0.0042 ppm after 24, 48 and 72 hrs, respectively. In combinatorial bioaasay ratio, 1:2 was observed to be more effective than other tested combinations with LC_{50} 0.0030, 0.0023 and 0.002 ppm after 24, 48 and 72 hrs of exposure period. Thus, the results revealed that the combination of Temephos and *A. flavus* demonstrated higher larvicidal activities indicating synergistic activity. Apart from raising new possibilities for *Culex* control it seems to be ideal approach to consider.

Keywords: *Aspergillus flavus;* Bioassay; *Culex quinquefasciatus;* Temephos; Synergism

1. Introduction
Mosquitoes are able to transmit numerous pathogens and parasites through their bites causing life threatening diseases to humans. *Culex quinquefasciatus* is painful and persistent biter responsible for one of the neglected tropical disease, Lymphatic filariasis (LF), caused by the lymphatic dwelling nematodes, *Wuchereria bancrofti* and *Brugia malayi*. It is a serious public health problem in India, where one third of global filarial cases occur, according to W.H.O. [26]. Over 120 million people are currently infected worldwide with about 40 million disfigured and incapacitated by the disease [19]. Mosquito management with the development of resistance to conventionally used synthetic insecticides has become difficult. Additionally over and injudicious use of synthetic insecticides in vector control has resulted into environmental hazards through persistence and accumulation of non-biodegradable toxic components in the ecosystem, toxic effects on human health and non-target organisms. These problems have prompted researchers to look for alternative insecticidal agents with high efficacy and low or no adverse effects to environment and human health.
Synergism is the joint action where one component of the mixture has the effect of increasing potency of the other component of the mixture such that their combined effect is greater than the amount of their individual effects. Some insecticides have the capacity to increase stress and affect insect behavior which may lead to improved performance of entomopathogens [21]. Entomopathogenic fungi are among the most important microbes considered as potential biological control agents [14]. They are preferred as they exhibit selective toxicity, do not persist, and do not need to be ingested as other microbes [17]. Synergistic effects between entomopathogenic fungi and synthetic pesticides have therefore, been studied for enhanced bioefficacy and solving the fungal related problems. Synergists are considered straight forward tools for overcoming metabolic resistance and their use could result in more effective control than the individual components of the mixture [23]. Synergistic mixture of compounds will provide similar control at reduced concentrations of the two compounds relative to individually applied compounds. Thus, results in reduction in cost and mammalian & non-target organism toxicity [4]. Reason behind the strategy is based on the probability that if resistance to one of the two insecticides is a rare and independent event, then the probability that resistance will occur simultaneously to both insecticides of the mixture is extremely low [5].

Thus, synergism has been preferred as an ideal strategy for resistance related problems and as an eco-friendly and economical control method as it reduces the quantity of insecticide needed to kill the target organisms. The objective, therefore, of the present study is to evaluate the synergistic action of Temephos and an entomopathogenic fungus, *A. flavus Cx. quinquefaciatus*, the filarial vector of India and other Asian pacific countries.

2. Materials and Method

The present study was assessed in the Applied Entomology & Vector Control Laboratory, Agra, India, during 2013 and 2014.

2.1. Test organisms

Mosquito colonies were reared and maintained in the laboratory, continuously at 27±2 ºC and 70-80% relative humidity (RH) under a photoperiod of 14:10 h (light/dark) without exposure to pathogens or insecticides. The larvae were fed with powdered brewer's yeast. Freshly molted 3rd instar larvae were continuously available for the mosquito larvicidal experiments.

2.2. Isolation of Fungus

A. flavus is a sporophyte and a haploid filamentous fungus. It is found all over the world, mainly at the warm places and is also abundant in areas with temperate climates during warm drought years. It grows at a temperature of 25 – 42 ºC and the optimum temperature for its growth is 37 ºC. It is yellow-green mold with distinctive conidiophore composed of a long stalk supporting an inflated vesicle.

The strain MTCC No. 1973 was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India and stored at 4 °C. Prior to testing against the mosquito larvae fungus were cultured on Peptones (20 g/L), dextrose (40 g/L), potato dextrose agar (PDA: 20 g/L) petri plates separately. The petriplates were placed in biological oxygen demand (BOD) incubator and held for 7 days. After 7 days, *Aspergillus* isolates were subcultured on Czepak solution agar media (sucrose 30 g/L, agar 15 g/L, $NaNO_3$ 2 g/L, K_2HPO_4 1g/L, KCl 0.5 g/L, $MgSO4.7H_2O$ 0.5 g/L, and $FeSO_4.7H_2O$ 0.01 g/L, at pH 7.3±0.2) to obtain pure cultures. *Aspergillus* species was determined morphologically under a microscope and isolates were stored at 4 ºC for further analysis [15].

2.3. Extraction of toxins

Isolate of *A. flavus* was cultured in 500 mL Erlenmeyer flasks containing 200 mL of sterile yeast extract sucrose (YES) liquid medium (20% sucrose and 5% yeast extract). The flasks were incubated separately for 7-10 days in the dark at 27-30 ºC without agitation. To lyse cells 25 mL of chloroform was added to recover mycelia and then agitated for 10 min on a rotator shaker (Biocraft). The flasks contents were filtered through Whatman no. 1 filter papers and the filtrate was used for toxin extraction. The filterate was transferred quantitatively to a separating funnel and extracted successively with 100 mL of chloroform to separate chloroform and aqueous layers. The procedure was repeated three times with lower transparent chloroform layer collected in a new flask. The chloroform was evaporated at 100 ºC by a vaccum rotatory evaporator to obtain the crude residue extract of each fungus [16]. The extracts were finally weighed and kept in refrigerator at 4 ºC until further use.

2.4. Bioassays

2.4.1. Synthetic Pesticide and Fungal extract

Bioassays were conducted according to W.H.O. standard protocol [27]. Temephos was diluted to obtain stock solutions of 10 ppm by dissolving 0.01 mL of Temephos in 1000 mL of dechlorinated tap water. Different working test concentrations of 250 mL ranging from 0.002 to 0.01 ppm were prepared in 500 mL capacity of Borosil glass beakers by diluting the stock solution for the exposure to mosquito larvae. Twenty, 3rd instar mosquito larvae, *Cx. quinquefasciatus* were exposed to each working concentration independently. The experiments were conducted in three replicates with a control experiment parallel with no pesticide. In each concentration larvae were fed with powdered brewer's yeast. The dead and moribund larvae were recorded as larval mortality by observing the movement of the larvae after each treatment period. The larvae were touched gently with the help of a glass rod and considered dead if they show no sign of movements and moribund if they moved a little but did not show any kind of swimming movement. The moribund larvae were considered dead as these larvae could never revive. Mortality was recorded 24, 48 and 72 hrs post-exposure separately. The mortality data were then subjected to probit analysis [8] to calculate LC_{50} and LC_{90} values with other statistical analysis.

The crude extract of Fungus (2.5 g) was diluted in 50 mL ethanol to get the stock solution of 50, 000 ppm. A range of working test concentrations were prepared by further diluting the stock solution using ethanol as a solvent and the controls were exposed to the solvent, i.e., ethanol alone. The bioassay was conducted with the same procedure depicted as above.

2.4.2 Temephos and fungal extract mixtures

10 ppm stock solutions of Temephos and *A. flavus* extract were prepared separately. Keeping temephos as the standard, its stock was mixed with the stock of fungal extract in ratios of 1:0.5, 1:1, 1:2 and 1:4. A range of desired test concentrations for each mixed formulation ratio were prepared by further diluting the combination in water. The mortality data were recorded after 24, 48 and 72 hrs of exposure and the larvicidal efficacy of each formulation were observed as above method.

2.5. Statistical analysis

Mortality data obtained for the Temephos and the fungal extract alone and for the mixed applications were analyzed by Probit Analysis [8] using MS Excel Fortran program to obtain regression equation and LC_{50} and LC_{90} values at 95% confidence limits. The synergistic factors (SF) for the mixed formulations were also calculated after calculating LC_{50} and LC_{90} for each combination [13].

$$\text{Synergistic Factor (SF)} = \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with fungal extract}}$$

SF value > 1; indicates synergism and
SF value < 1; indicates antagonism

3. Results

Table 1 reveals the larvicidal effect of Temephos and *A. flavus* against *Cx. quinquefasciatus.* For Temephos the LC_{50} values were 0.0060, 0.0055 and 0.0042 ppm after 24, 48 and 72 hrs of

exposure. The LC_{90} were 0.018, 0.016 and 0.011 ppm after 24, 48 and 72 hrs of treatment, respectively. The larvicidal toxicity of fungal extract of *A. flavus* shows LC_{50} values 13.62, 14.35 and 10.03 ppm after 24, 48 and 72 hrs of exposure. The LC_{90} were 70.31, 67.47 and 45.69 ppm after 24, 48 and 72 hrs of accordingly.

Table 1: Larvicidal potentiality of Temephos and *A. flavus* against, *Cx. quinquefasciatus*

Substances tested	Exposure period (Hours)	Regression equation	Chi-square	LC_{50} ±SE (ppm) (95% CL)	LC_{90} ±SE (ppm) (95% CL)
Temephos	24	2.73x+8.33	1.35	0.0060±0.0012 (0.0083-0.0038)	0.018±0.0085 (0.034-0.0011)
	48	2.72x+8.41	2.08	0.0055±0.0010 (0.0076-0.0035)	0.016±0.0076 (0.031-0.0016)
	72	2.99x+9.10	0.56	0.0042±0.0006 (0.0054-0.0030)	0.011±0.0038 (0.019-0.0037)
A .flavus	24	1.79x+1.16	7.86	13.62±2.35 (18.23-9.00)	70.31±29.02 (127.18-13.44)
	48	1.91x+0.89	8.09	14.35±2.39 (19.05-9.64)	67.47±26.10 (118.63-16.31)
	72	1.95x+1.11	10.85	10.03±1.51 (12.99-7.06)	45.69±14.59 (74.28-17.09)

CL, Confidential Limit

The larvicidal effect of Temephos and *A. flavus* at different doses against *Culex* larvae is presented in Table 2 and Figures 1 and 2. The bioassay results revealed that ratio 1:2 was the most promising larvicidal activity with the LC_{50} and LC_{90} 0.0030 and 0.0069 ppm after 24 hrs, 0.0023 and 0.0105 ppm after 48 hrs and 0.002 and 0.010 ppm after 72 hrs of post-exposure, respectively. The synergistic factor values of LC_{50} were 2, 2.39 and 2.1 at 24, 48 and 72 hrs.

Table 2: Larvicidal potentiality of Temephos in combination with *A. flavus* toxins *Cx. quinquefasciatus*

Temephos +*A. flavus* (Ratios)	Exposure period (Hours)	Regression equation	Chi-square	LC_{50} ±SE (ppm) (95% CL)	SF	Type of action	LC_{90} ±SE (ppm) (95% CL)	SF	Type of action
1:0.5	24	1.77x+6.85	1.85	0.0091±0.0014 (0.118-0.0063)	0.66	A	0.048±0.169 (0.081-0.014)	0.37	A
	48	1.62x+6.90	6.89	0.0067±0.0011 (0.0088-0.0045)	0.75	A	0.041±0.015 (0.071-0.011)	0.39	A
	72	1.82x+7.32	4.19	0.0053±0.0008 (0.0069-0.0037)	0.79	A	0.026±0.0079 (0.042-0.011)	0.42	A
1:1	24	1.37x+6.09	13.39	0.016±0.0034 (0.023-0.0094)	0.37	A	0.138±0.067 (0.269-0.0071)	0.13	A
	48	1.29x+6.17	15.79	0.012±0.0026 (0.017-0.0072)	0.46	A	0.122±0.0602 (0.240-0.0042)	0.13	A
	72	1.24x+6.21	21.79	0.010±0.002 (0.015-0.006)	0.42	A	0.114±0.057 (0.226-0.002)	0.09	A
1:2	24	3.55x+10.39	13.47	0.0030±0.0003 (0.0035-0.0023)	2	S	0.0069±0.0013 (0.0095-0.0043)	2.61	S
	48	1.97x+8.21	23.76	0.0023±0.0003 (0.0031-0.0016)	2.39	S	0.0105±0.0043 (0.0189-0.0020)	1.52	S
	72	1.74x+7.98	18.72	0.002±0.0004 (0.003-0.001)	2.1	S	0.010±0.005 (0.020-0.0009)	1.1	S
1:4	24	1.85x+7.46	14.98	0.0046±0.0006 (0.0059-0.0034)	1.30	S	0.023±0.0060 (0.035-0.011)	0.78	A
	48	1.32x+6.93	19.89	0.0035±0.0007 (0.0048-0.0021)	1.57	S	0.032±0.012 (0.057-0.0076)	0.5	A
	72	1.57x+7.57	23.96	0.002±0.0004 (0.003-0.001)	2.1	S	0.015±0.004 (0.023-0.007)	0.73	A

CL: Confidential Limits; SF, Synergistic factor; S, synergism; A, antagonism

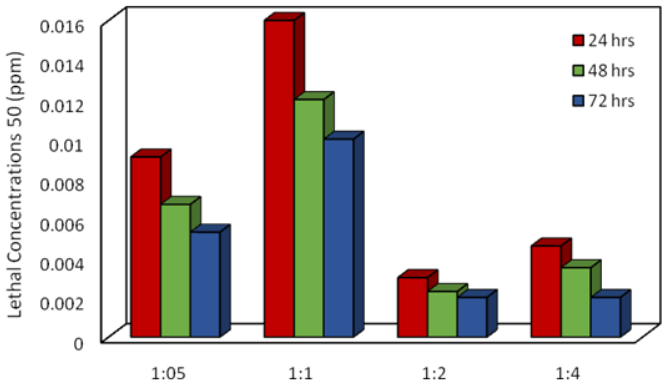

Fig 1: Comparative larvicidal potentiality of different ratios of Temephos and *A. flavus* against *Cx. quinquefasciatus* **(**Lethal Concentration LC_{50})

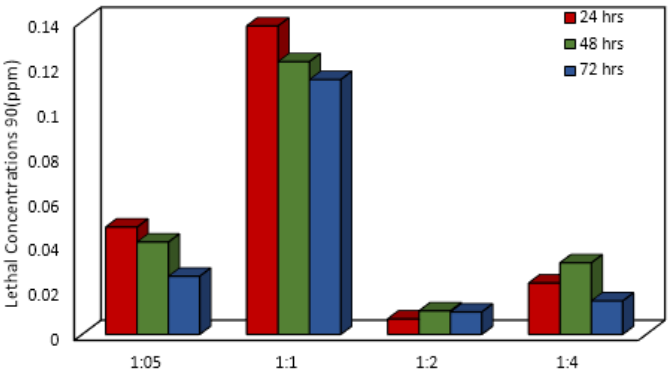

Fig 2: Comparative larvicidal potentiality of different ratios of Temephos and *A. flavus* against *Cx. quinquefasciatus* **(**Lethal Concentration LC_{90})

4. Discussion

In the present study, the treatment of *A. flavus* extract against *Culex* larvae showed considerable mortality. Fungal extracts have previously been tested for mosquito control by several workers. Govindarajan *et al.* [9] evaluated the larvicidal effect of 5 different fungi *viz., A. flavus, A. parasticus, Penicillium falicum, Fusarium vasinfectum* and *Trichoderma viride* against, *Cx. quinquefasciatus*. The LC_{50} values of *A. flavus, A. parasiticus, P. falicum, F. vasinfectum* and *T. viride* with 38.34, 40.39, 44.97, 50.03 and 54.16 mg/L, respectively. Among the five was found *A. flavus* to be the most toxic. Mishra *et al.* [18] observed larvicidal potentiality of secondary metabolites from 350 fungi and 94 actinomycetes against *Cx. quinquefasciatus, Anopheles stephensi and Aedes aegypti*. Among them, 133 fungal metabolites and 35 from actinomycetes were found effective. Vijayan & Balaraman [25] reported the larvicidal screening of fungal metabolites of 17 fungi against three major mosquitoes, *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti*. Maurya *et al.* (2011) [17] evaluated the larvicidal potential of certain fungi pathogens, *A. flavus, A. niger, A. parasiticus, F. sporotrichoides* and *P. verrucosum* against *Cx. quinquefasciatus* and *An. stephensi*. They found *A. flavus* with highest larvicidal bioefficacy having LC_{50} values of 9.54 and 10.98 ppm against *An. stephensi* and *Cx. quinquefasciatus*. Likewise, Thomas and Read [24] reported that the fungal derivatives were highly toxic against mosquitoes, while demonstrating low levels of toxicity to non-target organisms.

In terms of applications of mixtures, the extract of *A. flavus* was found to act either synergistically as well as antagonistically with the co-applied synthetic insecticide. The comparative evaluation of different combinations revealed that the ratio 1:2 was the most effective among other, showing synergistic activity at LC_{50} and LC_{90} values. The synergistic effect could be attributed to the weakening the mosquito's immune system by temephos induced stress which facilitates infection of *A. flavus* to larvae, according to Hiramori and Nishigaki [11]. Moreover, this synergistic activity may lead to reduced applied amounts of Temephos, thus limiting insect resistance problems and environmental hazards.

The synthetic insecticide Temephos and the fungus *A. flavus* act onto mosquito larvae in a different mode of action. The use of mixtures of these compounds could improve their joint action acting on different targets diminishes the short-term risk that resistance will arise to one or the other of the active compounds [5].

The findings of the present work are comparable to the insecticidal synergistic effects of entomopathogenic fungi with synthetic insecticides that have been reported by other researchers. Anderson *et al.* [1] evaluated the effects of combinations of *Beauveria bassiana* with insecticides against Colorado potato beetle. Synergistic effect of Imidacloprid and two entomopathogenic fungi against *Diaprepes abbreviates* larvae were investigated by Quintela and McCoy [20]. Kaakeh *et al.* [12] observed the toxicity of imidacloprid with *Metarhizium anisopliae* against German cockroach. Hiramori *et al.* [10] evaluated the combination of *M. anisopliae* with synthetic pesticide against *Anomala cuprea* larvae. Synergistic efficacy of fungal entomopathogens and permethrin against *An. gambiae* has been reported by Farenhorst *et al.* (2010) [7]. Cuthbertson *et al.* [6] reported the compatibility of the entomopathogenic fungus, *Lecanicillium muscarium* and insecticides for eradication of sweetpotato whitefly, *Bemisia tabaci*. Santos *et al.* [21] reported the toxicity of entomopathogenic fungi for use in combination with sub-lethal doses of imidacloprid for the control of leaf cutting ant, *Atta sexdens rubropilosa*. Seye *et al.* [22] evaluated *M. anisopliae* with neem oil against *An. gambiae* and *Cx. quinquefasciatus* adults. The present work is supported by the findings of the previous studies that reported the larvicidal potentiality of Temephos and *A. flavus* against *An. stephensi and A. flavus* with the most potent phyto extract (Petroleum ether extract) of *Cuscuta reflexa* against the larvae, *An. stephensi* and *Cx. quinquefasciatus* [2, 3].

The study concluded that selection of appropriate combinations of insecticides and fungal extracts for application as mixtures offers the prospect for effective mosquito control reducing environmental hazards, costs and resistance problems derived from the continuous use of conventional insecticides.

5. Acknowledgments

The authors are grateful to the Director of the institute and Head, Department of Zoology. We extend our thanks to district malaria office, Agra for providing Temephos for this work. First author is also grateful to University Grants Commission for financial support this work.

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