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Larvicidal toxicity of Temephos and entomopathogenic fungus, *Aspergillus flavus* and their synergistic activity against malaria vector, *Anopheles stephensi*

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ABSTRACT

Anopheles stephensi L. is the primary vector of malaria, infecting more than 500 million humans each year. Present study evaluated the compatibility of the organophosphate insecticide, temephos and mosquito-pathogenic fungus, *Aspergillus flavus* against third instar larvae of *An. stephensi.* Toxicity was assessed in different combination ratios, 1:1, 1:2 and 1:4. All the experiments were conducted according to WHO standard procedure. The mortality data were recorded after 24 and 48hrs of treatment. Fungal infection increased temephos induced mortality rate at 1:1 as compared to other ratios. The LC_{50} values of 1:1 were 0.0015 and 0.0011 ppm and LC_{90} values with 0.0045 and 0.0019 ppm after 24 and 48 hrs of post exposure, respectively. Combining *A. flavus* with temephos induced a higher impact on mosquito survival than the use of these control agents alone. The observed synergistic efficacy shows the potential for integrated fungus-insecticide control measure to dramatically reduce malaria transmission due to enhanced larval mortality and enable control at more moderate levels of coverage even in areas where insecticide resistance has rendered essentially ineffective.

Keywords: Organophosphate insecticide, Temephos, *Aspergillus flavus*, *Anopheles stephensi*, Synergism.

1. Introduction

Vector control is an essential requirement in control of epidemic diseases such as malaria, filariasis, dengue, yellow fever etc. that are transmitted by different species of mosquitoes. Malaria vector, *An. stephensi* continues to be the most worrisome disease transmitting vector. In India, malaria is transmitted by six vector species, in which *An. stephensi* is generally an active vector in urban areas as studied by Senthilkumar *et al.* [1]. In 2012, there are 99 countries and territories with ongoing malaria transmission and 5 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic according to WHO, [2]. In India, an estimated 2–3 million malaria cases and about 1,000 deaths were reported every year as observed by Lal *et al.* [3]. The indiscriminate uses of chemical insecticides have adverse effects based on intervention measures for the control of mosquito vectors. This has received wide public apprehension because of several problems like insecticide resistance, resurgence of pest species, environmental pollution, toxic hazards to humans and non-target organisms. These problems have necessitated the need to explore and develop alternative strategies using eco-friendly, bio-degradable bio-products which are less toxic to non-target organisms too.

There is a resurgence of interest in the use of bio-pesticides and among them entomopathogenic fungi provide a possible additional tool for the control of insecticide-resistant mosquitoes. They are ideal for IPM programme because they are relatively safe to use and have a narrower spectrum of activity than chemical insecticides Lacey & Goettel, [4] and Keller, [5]. They are considered among the most important microbes as the source of potential biological control agents as described by Keller, [5] and Srivastava *et al.* [6]. Entomopathogenic fungi are preferred as they exhibit selective toxicity, do not persist, and do not need to be ingested as other microbes as reported by Maurya *et al.* [7] and have low toxicity to non-target organisms. There is worldwide interest in the use of entomopathogenic fungi as biological control agents, and a significant advance in development and manufacturing of these agents in the future is expected with recent biotechnological innovations as observed by Khachatourians [8].

We have screened extracellular secondary metabolites from *A. flavus* against mosquito larvae. Which are considered as diversed natural products synthesized by cells that have stopped dividing as reported by Govindarajan *et al.* [9].

Synergists are considered straight forward tools for overcoming metabolic resistance and could be more effective than the individual components of the mixture. Thus, synergism has been preferred as an ideal strategy for resistance related problems, eco-friendliness and economical as it reduces the quantity of insecticide needed to kill the target population than the individual components of mixture. In this context, the present bioassay was carried out to observe the larvicidal efficacy of the combinations of temephos and *A. flavus* against larvae of *An. stephensi*, the major malaria vector in India and other sub-tropical countries with the aim of developing joint action larvicide as supplementary and complementary measures for the management of mosquito vectors.

2. Materials & Methods

The study was conducted in the Applied Entomology & Vector Control Laboratory, Agra, India, during 2011and 2012.

2.1 Mosquito Rearing

The malarial vector, *An. stephensi* was reared in our laboratory, maintained continuously at 27±2 ºC and 70-80% relative humidity 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 third instars of *Anopheline* larvae were continuously available for the mosquito larvicidal bioassay.

2.2 Isolation of Fungus

A. flavus was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India (MTCC No. - 1973) and stored at 4 °C. Prior to testing on mosquito larvae it was cultured on Peptones (20 g/L), dextrose (40 g/L), potato dextrose agar (PDA: 20 g/L) petriplates 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 1 g/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 °C for further analysis according to Klich & Pitt [10].

2.3 Extraction of toxins

Isolates of *A. flavus* were cultured in 500 mL Erlenmeyer flasks containing 250 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 °C without agitation. To lysed cells 25 mL of chloroform were added to recover mycelia and then agitated for 10 min on a rotator shaker. The flasks contents were filtered (Whatman no. 1) and the filtrate was used for toxin extraction. The filtrate 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 °C by a vacuum rotary evaporator to obtain the crude extract of each fungus according to Mallek *et al.* [11]. The extract was finally weighed and kept in refrigerator at 4 °C until further use.

2.4 Bioassay of Temephos and *A. flavus*

Bioassay of fungus, *A. flavus* and synthetic insecticide, temephos was repeated and confirmed their bioefficacy already tested in our lab, Applied Entomology and Vector Control Labs by Maurya *et al.* [7] and Mohan *et al.* [12]. The stock solution of 10 ppm was prepared by dissolving 0.01 mL of temephos in 1000 mL of deionised 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 larvae exposure. Twenty, 3rd instar larvae were exposed to each working concentration independently. The experiments were conducted in three replicates with a control parallel. The dead and moribund larvae were recorded as larval mortality. The mortality of larvae was determined by observing the movement of the larvae after the treatment period. The larvae were touched gently with the help of a glass rod and considered dead if they showed no sign of movements. The larvae were considered 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 observations were recorded after 24 and 48 hrs of exposure. All the experiments were devised according to WHO standard procedure [13].

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

2.5 Combinatorial bioassay

For combinatorial studies, 10 ppm stock solution of temephos and *A. flavus* extract were prepared. Keeping synthetic insecticide, temephos as standard, its stock was mixed with the stock of fungal extract in ratios of 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 larvicidal bioassay and mortality data of each ratio were recorded after 24 and 48 hrs of exposure with same procedure as depicted above.

2.6 Statistical analysis

The average larval mortality data obtained for the individual bioassay of temephos and the fungal extract and for the combinatorial studies were subjected to probit analysis according to Finney [14] for calculating LC_{50}, LC_{90}, standard error, regression equation, chi- square values and lower fiducial limit and upper fiducial limit at 95% confidence limits. The co-toxicity coefficient, Sarup *et al.* [15] and synergistic factor, Kalayanasundaram & Das [16] for the mixed formulations were also calculated after calculating LC_{50} and LC_{90} for each combination.

$$\text{Co-toxicity coefficient} = \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with fungal extract}} \times 100$$

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

Value of SF > 1 indicates synergism and SF<1 indicates antagonism

3. Results

3.1 Bioefficacy of temephos and *A. flavus*

Table 1 provides larval mortality of *An. stephensi* after the

treatment of synthetic insecticide, temephos and ethanolic extract, *A. flavus* individually (Figure 1, 2). The larvicidal potentiality of temephos against *An. stephensi* represented the LC_{50} and LC_{90} values as follows: LC_{50} value was 0.0025 and 0.0023 ppm after 24 and 48 hrs of exposure, respectively. The LC_{90} value was 0.0052 and 0.0040 ppm after 24 and 48 hrs of treatment, respectively.

The larvicidal potentiality of fungal extract, *A. flavus* against *An. stephensi* represented the LC_{50} and LC_{90} values as follows: LC_{50} value was 10.872 and 8.153 ppm after 24 and 48 hrs of exposure, respectively. The LC_{90} value was 33.233 and 27.286 ppm after 24 and 48 hrs of exposure, respectively.

Table 1: Larvicidal potentiality of temephos and *A. flavus* against, *Anopheles stephensi*

***A.flavus* and Temephos**	**Exposure period (Hours)**	**Regression equation**	**Chi-square**	**LC_{50} ±SE (Fiducial limits ppm)**	**Relative toxicity**	**LC_{90} ±SE (Fiducial limits ppm)**	**Relative toxicity**
Temephos	24	4.089x+11.544	0.206	0.0025±0.0004 (0.0032-0.0018)	4348.8	0.0052±0.0009 (0.0070-0.0033)	6390.96
	48	5.258-13.606	0.609	0.0023±0.0003 (0.0029-0.0017)	4726.96	0.0040±0.0006 (0.0051-0.0029)	8308.25
A. flavus	24	2.642-7.978	0.187	10.872±2.019 (14.830-6.913)	1.00	33.233±14.572 (61.784-4.662)	1.00
	48	2.443-0.331	0.826	8.153±1.857 (11.793-4.513)	1.33	27.286±11.306 (49.446-5.125)	1.22

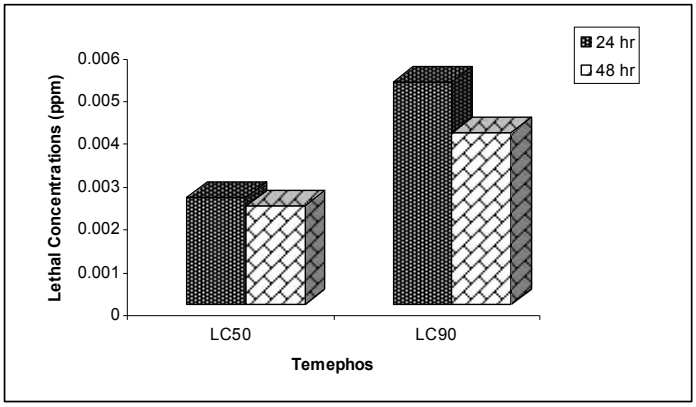

Fig 1: Lethal concentrations of temephos against *Anopheles stephensi* at 24 and 48 hrs of exposure periods.

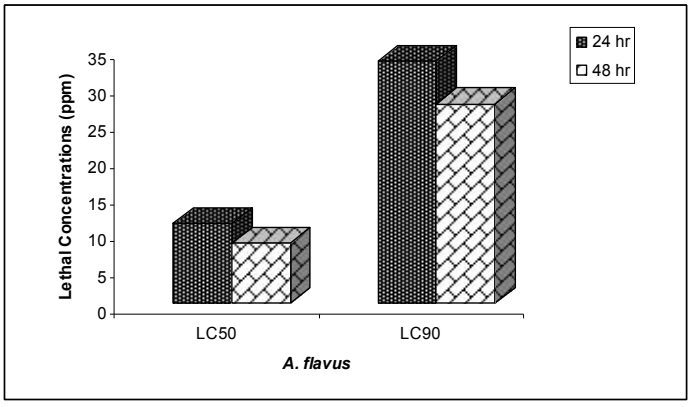

Fig 2: Lethal concentrations of *A. flavus* against *Anopheles stephensi* at 24 and 48 hrs of exposure periods.

3.2 Combined Bioefficacy

In order to detect potential synergistic effects between temephos and *A. flavus* against anopheline larvae, we evaluated three different ratios 1:1, 1:2 and 1:4 as depicted in Table 2 (Figure 3. A & B). Synergistic factor has been worked out and the highest synergism was found to be in 1:1 as compared to 1:2 and 1:4. The ratio 1:1 had LC_{50} value 0.0015 and 0.0011 ppm after 24 and 48 hrs of exposure, respectively. LC_{90} value was 0.0045 and 0.0019 ppm after 24 and 48 hrs of exposure, respectively. The LC_{50} value for ratio 1:2 was 0.0029 and 0.0011 ppm after 24 and 48 hrs of exposure, respectively. The LC_{90} value was 0.0133 and 0.0020 ppm after 24 and 48 hrs of exposure, respectively. For ratio 1:4 the LC_{50} value was 0.0057 and 0.0012 ppm after 24 and 48 hrs of exposure, respectively. The LC_{90} value was 0.0430 and 0.0020 after 24 and 48 hrs of exposure, respectively.

A

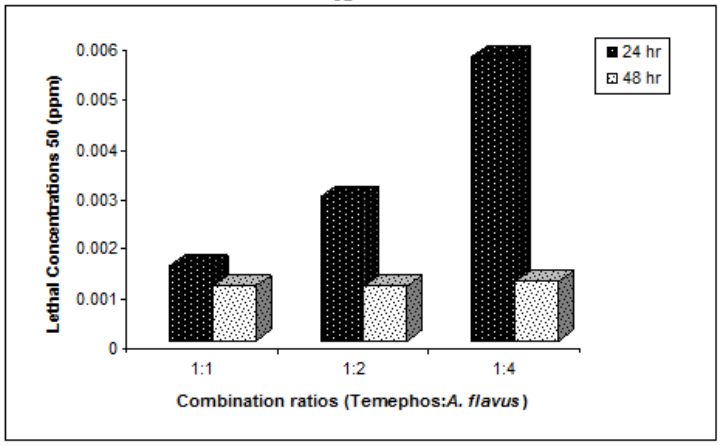

Table 2 also illustrates the co-toxicity coefficient and the synergistic factors of these ratios. The co-toxicity coefficient for the 1:1 were 166.67 and 209.09 with synergistic factor 1.667 and 2.091 at 24 and 48 hrs respectively for the LC_{50} which indicates synergism at both the exposure periods and with the LC_{90} co-toxicity coefficient was 115.55 and 210.53 with synergistic factor 1.155 and 2.105 showing synergism at 24 and 48 hrs respectively with synergistic action in both the cases. For ratio 1:2, the co-toxicity coefficient values at LC_{50} were 86.207 and 209.09 with synergistic factor 0.862 and 2.091 shows antagonism after 24 hrs and synergism after 48 hrs of exposure respectively.

Table 2: Larvicidal potentiality of temephos in combination with *A. flavus* toxins against *Anopheles stephensi*

Ratio	Exposure period (Hours)	Regression equation	Chi-square	LC_{50}±SE (Fiducial limits ppm)	Co-toxicity coefficient	Synergistic factor	Type of action	LC_{90}±SE (Fiducial limits ppm)	Co-toxicity coefficient	Synergistic factor	Type of action
1:1	24	2.861x+10.133	3.072	0.0015±0.0003 (0.0021-0.0011)	166.67	1.667	S	0.0045±0.0012 (0.0068-0.0022)	115.55	1.155	S
	48	5.267x+15.470	1.214	0.0011±0.0001 (0.0013-0.0008)	209.09	2.091	S	0.0019±0.0004 (0.0027-0.0011)	210.53	2.105	S
1:2	24	1.936x+7.978	1.700	0.0029±0.0007 (0.0042-0.0016)	86.207	0.862	A	0.0133±0.0046 (0.0222-0.0043)	39.097	0.391	A
	48	5.209x+15.124	2.061	0.0011-0.0001 (0.0014-0.0009)	209.09	2.091	S	0.0020±0.0004 (0.0029-0.0011)	200	2	S
1:4	24	1.456x+6.815	1.588	0.0057±0.0023 (0.0102-0.0011)	43.860	0.438	A	0.0430±0.0203 (0.0828-0.0032)	12.094	0.121	A
	48	5.733x+16.003	1.959	0.0012±0.0001 (0.0015-0.0009)	191.67	1.917	S	0.0020±0.0004 (0.0028-0.0012)	200	2	S

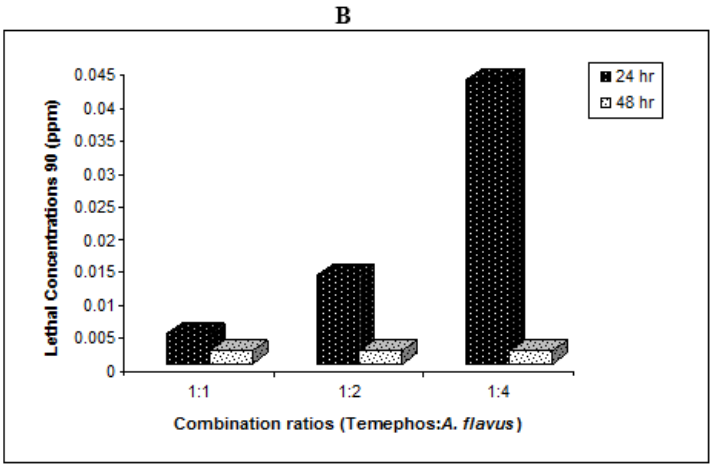

Fig 3: Comparative larvicidal potentiality of temephos and its combination with *Aspergillus flavus* against *Anopheles stephensi* A. Lethal Concentration LC_{50} and B. Lethal Concentration LC_{90}

The co-toxicity coefficient values at LC_{90} were 39.097 and 200 with synergistic factor 0.391 and 2 which shows antagonism after 24 hrs and synergism after 48 hrs of exposure respectively. For ratio 1:4, the co-toxicity coefficient was 43.860 and 191.67 with synergistic factor 0.438 and 1.917 at LC_{50} and shows antagonism after 24 hrs and synergism after 48 hrs of exposure respectively. The LC_{90} had the co-toxicity coefficient 12.094 and 200 with synergistic factor 0.121 and 2 which shows antagonism after 24 hrs and synergism after 48 hrs of exposure respectively.

4. Discussion

The present study demonstrated larvicidal activity of temephos and *A. flavus* and synergistic effects of their different combinations against anopheline larvae. In comparative evaluation of combinations the ratio 1:1 shows the optimum synergistic activity with LC_{50} 0.0015 and 0.0011 ppm after 24 and 48 hrs of exposure which could allow for reduction in temephos concentration used for the mosquito larvae control that is with LC_{50} 0.0025 and 0.0023 ppm and *A. flavus* concentration with LC_{50} 10.872 and 8.153 ppm after 24 and 48 hrs of exposure when used individually. There by restriction on insecticide resistance as well as other negative environmental impacts. This synergistic effect is presumably due to the combination of temephos and *A. flavus* together weakening the mosquito's immune system by reinforcing their combined impact as studied by Hiramori & Nishigaki *et al.* [17]. Findings of our combinatorial studies has been supported by various workers who reported that mixtures of more than one insecticide with different modes of action are proving to be effective and recommended for integrated resistance management in some insect pests as observed by Tang & Huang [18], Shen & Wu [19] Zhang *et al.* [20]. Seye *et al.* [21] evaluated *Metarhizium anisopliae* with neem oil against *Anopheles gambiae* and *Culex quinquefasciatus* adults in Sengal and found adult's agitation followed by rapid knockdown. Synergistic efficacy of fungal entomopathogens and permethrin against, *An. gambiae* was reported by Farenhorst *et al* [22].

In our studies, the combined treatment of fungus and insecticides caused the granular cells to decrease. Hemocytes of larvae were significantly affected by a combined action of *A. flavus* and insecticides. The results indicated that the synergism might be caused by the inhibitor of the larvae cellular immune system. Furthermore, phenoloxidase activity of mosquito larvae was inhibited by the mixed application of fungus and insecticides. Melanization depending on the activation of the PO cascade is one of the major defenses of the humoral reaction against non-self as observed by Wago [23]. Results also showed that the synergism might be caused by the inhibition of the larval humoral defense system.

Presumably the observed synergism in our studies is higher at lower doses due to combined impact of these two complementary mixtures simultaneously acting in different ways. However, the reason particularly to explain the decrease of synergism at higher concentrations is still not clear as also stated by Corbel *et al.* [24]. Furthermore, our work has been comparable with work of many researchers who examined the synergism in mixtures of synthetic insecticides and fungi against various pests. Boucias *et al.* [25] showed that the synergistic effect of *Beauveria bassiana* and imidacloprid on the termite *Reticulitermes flavipes*. Hiromori and Nishigaki [17] founded synergism between *M. anisopliae* and fenitrothion or teflubenzuron against scarab beetle larvae. Hornbostel *et al.* [26] investigated that combination of permethrin with *M. anisopliae* was highly effective in *I. scapularis* control options by inducing the highest mortality (approximately 90%). Anderson *et al.* [27] evaluated the effects of combinations of *B. bassiana* with insecticides against Colorado potato beetle. Synergistic effect of Imidacloprid and two entomopathogenic fungi against *Diaprepes abbreviatus* larvae were investigated by Quintela and McCoy [28]. Kaakeh *et al.* [29] observed the toxicity of imidacloprid with *M. anisopliae* against German cockroach. Hiramori *et al* [30] evaluated the combination of *M. anisopliae* with synthetic pesticide against *Anomala cuprea* larvae. Cuthbertson *et al.* [31] reported the compatibility of the entomopathogenic fungus, *Lecanicillium muscarium* and insecticides for eradication of

sweetpotato whitefly, *Bemisia tabaci*. Santos *et al.* [32] 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.* Synergistic effect of some entomopathogenic fungi and synthetic pesticides, against two spotted spider mite, *Tetranychus urticae* were studied by Amjad *et al.* [33].

Our results concluded that the combined use of *A. flavus* and temephos against malarial vector shows synergistic activity with reduced the quantity of insecticide and increased their activity. This indicates that the combined interventions may considerably improve mosquito control programme than the single interventions of insecticides for mosquito management. However, further study is needed to develop more synergists that could be utilized in mosquito management programme to reduce the indiscriminate use of harmful chemical insecticides.

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