BIOEFFECTIVENESS OF NANOENCAPSULATED ASPERGILLUS FLAVUS AND CUSCUTA REFLEXA COMBINATION AGAINST ANOPHELINE AND CULICINE LARVAE

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Abstract: The present work assessed the larvicidal toxicity of nanoencapsulated mixture, Aspergillus flavus and Phyto extract, Cuscuta reflexa against 3rd instar larvae of Anopheles stephensi and Culex quinquefasciatus. The nanoparticles were encapsulated by simpler and economical melt-dispersion method in different ratios viz., 1%-8% within a polymer, Polyethylene glycol. Nanoparticles were in the size range of 100-200 nm. Larvicidal bioassays showed that nanoformulation with 2% mixture has a greater larvicidal effect, with LC50 11.16, 7.07 and 1.83 mg/L against anopheline larvae and 13.37, 7.72 and 3.36 mg/L against culicine larvae after 24, 48 and 72 hrs of exposure. It was the most effective than other nanoformulations and non-encapsulated mixture. The data showed that the bioefficacy of 2% nanoformulation increases with the increase of time period. To minimize the hazardous effect of pesticides, the synthesized nanoencapsulated mixture are feasible with eco-friendly, effective and economical approach in mosquito management.

Keywords: Anopheles stephensi, Aspergillus flavus, Culex quinquefasciatus, Cuscuta reflexa, Nanoencapsulation, Polyethylene glycol

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INTRODUCTION

Mosquitoes are the principal vector of many vector-borne diseases affecting human beings and animals, causing millions of deaths every year. To prevent proliferation of mosquito borne diseases there is an emergent need to develop an environment friendly mosquito control approach for curtailing the vector menace responsible for the majority of tropical diseases.

Anopheles stephensi Liston (An. stephensi) is the primary vector of malaria caused by the protozoan parasites, belonging to the genus Plasmodium. Over 1.2 million global malaria deaths were reported in 2010, in both children and adults (Murray et al. 2012). 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 (2012). In India, an estimated 2–3 million malaria cases and about 1,000 deaths were reported every year as observed by Lal et al. (2010).

Culex quinquefasciatus Say (Cx. quinquefasciatus), is the main vector of bancroftian filariasis, considered as one of the major neglected diseases. Lymphatic filariasis caused by mosquito-transmitted filarial nematodes, including Wuchereria bancrofti and Brugia malayi. Approximately, 1.2 billion which is about 20% of the world’s population are at risk of acquiring infection, one third of them live in India, one third in Africa and most of the remainder in Asia, the Pacific and Americas (Kuppusamy and Murugan 2008). An estimated 1.4 billion people in 73 countries are threatened by lymphatic filariasis, commonly known as elephantiasis. Over 120 million people are currently infected, with about 40 million disfigured and incapacitated by the disease (WHO 2013).

Nanotechnology is an emerging field in the world of pesticides & pest control which involves synthesis and development of various nanopesticides. Biosynthesis of nanoparticles using bacteria (Prakash et al. 2011), fungus and plants (Soni and Prakash (2012) and Irvani et al. 2011) has emerged as a simple and viable alternative to more complex physical and chemical synthetic procedures to obtain nanomaterials. Nanoencapsulation is a process through which a chemical such as an insecticide is slowly but efficiently released to a particular host or target organism. Nanoencapsulation with nanoparticles in form of pesticides allow for proper absorption of the chemical into the target organism unlike the case of larger particles. Release mechanisms of nanoencapsulation include diffusion, dissolution, biodegradation and osmotic pressure with specific pH (Ding and Shah 2009; Vidhyalakshmi et al. 2009). Nanoencapsulation is currently the most promising technology for protection of host plants against insect pests. Now, most leading chemical companies focus on formulation of nanoscale pesticides for delivery into the target host tissue through nanoencapsulation (Prasad et al. 2014). Although having widespread use as controlled-release systems in medicine and
pharmacy, nanoparticles have found little usage in agriculture, particularly as carriers for pesticides (Kulkarni et al. 1999; Soppirmath and Aminabhavi 1999; Guan et al. 2008). Thus, there is a need for “green approach” that includes a clean, nontoxic, and environment-friendly method of nanoparticle synthesis (Mukherjee et al. 2001). Keeping the lacunae in view the present study attempted to examine the biogenesis of nanoencapsulated mixture of Cuscuta reflexa and Aspergillus flavus.

The genus Cuscuta (Family: Convolvulaceae) contains about 150 species and all are parasitic on angiosperms. It is commonly known as Amar bel (meaning, immortal vine) and is distributed throughout Ceylon and India. It is a perennial herb and is widely used as a valuable herbal drug in traditional medicine. It is an extensive parasitic vine related to the Morning glory family. It grows in a prolific manner over host plants (or other support) with inter-twined stems, giving it a common name of Devils Hair. The plant is leafless and rootless. The twining stem develops Haustoria which are root like and penetrate the host stem to draw water and nourishment. The flowers are small, white, having a perfect bell shape and a fleshy calyx, attached directly to the stem nodes. Used as anti-spasmodic, heamodynamic, anti-steroidogenic, anti-hypertensive, anti-inflammatory, anti-pyretic and laxative properties (Abdin et al. 2012). The entomopathogenic fungi provide a possible additional tool for the control of insecticide resistant mosquitoes and have low toxicity to non-target organisms (Govindrajan et al. 2005). They are ideal for IPM programme because they are relatively safe to use and have a narrower spectrum of activity than chemical insecticides. They are considered among the most important microbes as the source of potential biological control agents (Srivastava et al. 2011). Entomopathogenic fungi are preferred as they exhibit selective toxicity, do not persist, and do not need to be ingested as other microbes and have low toxicity to non-target organisms (Bhan et al. 2013a, b).

Materials and methods

Materials

C. reflexa (stems) were collected from different localities of Agra. Pure fungal strains of A. flavus (MTCC No-1973) strain was procured from the Institute of Microbial Technology, Chandigarh, India. Polyethylene glycol 6000 (PEG) was purchased from Merck.

Target organisms

The mosquito vectors, An. stephensi and Cx. quinquefasciatus were reared in the laboratory, maintained continuously at 27±2 °C 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 larvae were continuously available for the mosquito larvicidal experiments.
Isolation of Fungus

The obtained A. flavus strain was cultured on Peptones (20g/L), dextrose (40g/L), potato dextrose agar (PDA: 20g/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 30g/L, agar 15g/L, NaNO3 2g/L, K2HPO4 1g/L, KCl 0.5g/L, MgSO4.7H2O 0.5g/L, and FeSO4.7H2O 0.01g/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 (Klich & Pitt 1994)21.

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 were 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 vaccum rotatory evaporator to obtain the crude extract of each fungus (Mallek et al. 1993)22. The extracts were finally weighed and kept in refrigerator at 4 °C until further use.

Phytoextract Preparation

The stems of C. reflexa were collected from the different localities of Agra. The stems were than washed in running tap water and dried in the shade. The shade dried stems were crushed mechanically and subjected to extraction with petroleum ether in a soxhlet apparatus for 72 hrs. Extract was concentrated by removing the solvent by vaccum rotatory evaporator. The extract obtained as thick viscous paste was completely evaporated to dryness at room temperature and kept in refrigerator below 5 °C after weighing until further use.

Synthesis of non-capsulated combination

The pesticides were weighed and mixed in equal proportion (1:1). Mixture was diluted in ethanol to prepare the stock solution of 1000 mg/L. Different range of desired test concentrations for each mixed formulation ratio were prepared by further diluting the mixture with deionized water in 500 ml capacity of Borosil glass beakers for the exposure to mosquito larvae. The mortality data were recorded after 24, 48 and 72 hrs of exposure and the larvicidal efficacy of each formulation was observed as above said.
Synthesis of nanoencapsulated combination

The encapsulation of nanoparticles mixture was conducted by using melt-dispersion method (Peng et al. 2008)\textsuperscript{23}. PEG (6000) was heated in four parts (49.5, 49.0, 48.0, and 46.0 g) separately at 65 °C to these melted parts 0.5, 1, 2 and 4g of mixtures were added to obtain 1%, 2%, 4% and 8% of nanoparticles mixture. The mixed nanoformulations were stirred gently with the glass rod to ensure even distribution of the mixture. Each nanoformulation were cooled at room temperature and grounded completely in a mortar and sieved using a 200 mesh sieve. The nanopesticides were then placed in airtight, self-sealable polyethylene pouches and stored at 25 °C in desiccators containing calcium chloride to prevent moisture absorption prior to further experiments (Bhan et al. 2014)\textsuperscript{24}.

Bioassay of non-encapsulated combination

Twenty, 3\textsuperscript{rd} instar larvae, \textit{An. stephensi} and \textit{Cx. quinquefasciatus} were collected separately and placed in a 250 mL beaker with 200 mL of water and then transferred gently to different working test concentrations with a control individually. Distilled water without any pesticide added was kept as control. The experiments were arranged in triplicates at ambient temperature (27±2 °C) and 70-80% relative humidity. A small aliquot of yeast powder was supplied for nutrition. Daily loss of water from experimental series was adjusted by adding required quantity of tap water up to the marking on the experimental beakers. The larval mortality in both treated and controls were monitored after 24, 48 and 72 hrs of exposure. The larvae were considered dead if they were immobile and unable to reach the water surface (Macedo et al. 1997)\textsuperscript{25}.

Bioassay of nanoencapsulated combination

In order to evaluate the larvicidal efficacy of the encapsulated nanopesticide mixture, the experiments were carried out employing usual procedure as depicted above. A certain mass of NPs (50 mg) was placed in a 50 mL Beaker containing deionized water to prepare stock solution of 1000 mg/L independently. 20 third instar \textit{An. stephensi} and \textit{Cx. quinquefasciatus} larvae. Larvae population mortality was then determined after 24, 48 and 72 hrs by counting dead specimens which were subsequently removed. A control (blank) sample was used with the same nanoparticle composition and larvae number, however, with no pesticide loading present. All experiments were carried out in triplicates and the data were analysed.

Statistical analysis

The recorded mortality data after 24, 48 and 72 hrs of exposure period for each experiment was analysis by using probit analysis (Finney 1971)\textsuperscript{26}. Experiments with more than 20% mortality in control were discarded and if mortality ranging 5- 20% in control, the mortality data were corrected by applying Abbot’s formula (Abbot 1925)\textsuperscript{27} so as to remove the factors
other than the nanopesticides. The lethal concentration for 50% and 90% mortality (LC$_{50}$ and LC$_{90}$) values with other statistical values were determined at 95% fiducial confidence intervals along with relative toxicity and chi-square.

**Characterisation of nanoparticles**

**Transmission Electron Microscopy**

The morphology of the most potent nanoencapsulated form (2%) was determined by Transmission Electron Microscopy (TEM). For TEM studies, a small amount of nanoformulation was dissolved in deionised water. A drop of this solution was placed on a copper grid and allowed to dry in vacuum. Micrographs were obtained using Philips Morgagni (M-268).

**Particle size and distribution**

The nanoparticle size and distribution was analyzed with the Nanozetasizer (Malvern). Sample was diluted with deionised water with 0.5 g in 50 mL and filtered through a millipore filter to avoid any contamination. Each measurement was performed in triplicate.

**Results**

**Characterisation of nanoparticles**

The nanoparticles of the most effective encapsulated nanoformulation with 2% mixture were in the range of 100-200 nm and its size distribution has been showed in Fig. 1. The electron microscopic study of the nanoparticles using TEM revealed that the nanoparticles predominate with spherical morphology. Most of the nanoparticles were roughly shaped with smooth edges (Fig. 2).

![Figure 1 Nanoparticles size distribution of the most potent nanoencapsulated formulation.](image)
Figure 2 TEM micrograph of the most potent nanoencapsulated formulation.

Bioefficacy of non-encapsulated combination

The combinatorial bioassay of different combinatorial ratios of A. flavus with C. reflexa against anopheline larvae is represented in Table (1). The ratio 1:1 has the LC$_{50}$ value 7.910, 6.368 and 4.317 mg/L after 24, 48 and 72 hrs. The LC$_{90}$ values were 17.452, 15.525 and 15.627 after 24, 48 and 72 hrs of exposure. The combinatorial bioassay of different combinatorial ratios of A. flavus with C. reflexa against culicine larvae is also represented in Table (1). The ratio 1:1 has the LC$_{50}$ value 8.712 mg/L, 6.671 and 5.755 mg/L after 24, 48 and 72 hrs. The LC$_{90}$ values were 15.763, 12.993 and 11.781 mg/L after 24, 48 and 72 hrs of exposure.

Bioefficacy of nanoencapsulated combination

The data for the Larvicidal responses of different encapsulated nanoformulations of fungal extract, A. flavus with extract of C. reflexa against An. stephensi and Cx. quinquefasciatus were depicted in Table (2) and (3). The results reveal that the mortality rates of nanoformulation with 2% of mixture have the highest efficacy amongst all nano-formulations and the conventional forms tested against both the larvae. The data mentioned in Table (2) shows the LC$_{50}$ and LC$_{90}$ against anopheline larvae. The LC$_{50}$ for 1% nanoformulation were 46.13, 26.22 and 16.30 mg/L and LC$_{90}$ 163.94, 185.30 and 131.39 mg/L after 24, 48 and 72 hrs. In case of 2% nanoformulation, the LC$_{50}$ and LC$_{90}$ were 11.16, 7.07 and 1.83 mg/L and 77.04, 52.73 and 16.38 mg/L after 24, 48 and 72 hrs respectively. The LC$_{50}$ for 4% nanoformulation was 85.73, 67.01 and 56.29 mg/L after 24, 48 and 72 hrs. The LC$_{90}$ was 355.32, 300.19 and 261.80 mg/L after 24, 48 and 72 hrs, respectively. For 8% nanoformulation, the LC$_{50}$ was 202.62, 149.92 and 107.22 mg/L after 24, 48 and 72 hrs, accordingly. The LC$_{90}$ values were 1057.43, 853.69 and 639.06 mg/L after 24, 48 and 72 hrs.
The LC$_{50}$ and LC$_{90}$ of different nanoformulations against culicine larvae are mentioned in Table (3). For 1% nanoformulation the LC$_{50}$ were 52.06, 40.66 and 32.59 mg/L after 24, 48 and 72 hrs. The LC$_{90}$ values were 191.27, 146.23 and 131.63 mg/L after 24, 48 and 72 hrs, respectively. In case of 2% nanoformulation, the LC$_{50}$ values were 13.37, 7.73 and 3.36 mg/L. The values for LC$_{90}$ were 116.55, 59.70 and 16.88 mg/L after 24, 48 and 72 hrs. The LC$_{50}$ for 4% nanoformulation was 259.79, 227.36 and 200.85 mg/L after 24, 48 and 72 hrs of exposure period. The LC$_{90}$ were 931.46, 841.35 and 809.74 mg/L after 24, 48 and 72 hrs. For 8% nanoformulation, LC$_{50}$ and LC$_{90}$ was 213.03, 183.61 and 147.77 mg/L for the former and 942.67, 1058.42 and 794.08 mg/L for the later after 24, 48 and 72 hrs, accordingly.

**Table -1 Combinatorial toxicity of* A. flavus *with extract (Pee) of* C. reflexa *against anopheline and culicine larvae.**

<table>
<thead>
<tr>
<th>Target organisms</th>
<th>Exposure period (Hours)</th>
<th>Chi-square</th>
<th>Regression equation</th>
<th>LC$_{50}$ ±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (irrespect of exposure period)</th>
<th>LC$_{90}$ ±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (irrespect of exposure period)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. stephensi</em></td>
<td>24</td>
<td>1.68</td>
<td>3.73 x 2.08</td>
<td>7.91±0.97 (9.81-6.01)</td>
<td>1.10</td>
<td>17.45±3.85 (25.00-9.89)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.23</td>
<td>3.31 x 0.97</td>
<td>6.37±0.92 (8.18-4.55)</td>
<td>1.37</td>
<td>15.52±3.55 (22.48-8.57)</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.27</td>
<td>2.29 x 1.25</td>
<td>4.32±1.19 (6.65-1.98)</td>
<td>2.02</td>
<td>15.63±4.65 (24.74-6.51)</td>
<td>1.12</td>
</tr>
<tr>
<td><em>Cx. quinquefasciatus</em></td>
<td>24</td>
<td>5.54</td>
<td>4.98 x-4.66</td>
<td>8.71±0.88 (10.44-6.98)</td>
<td>1.00</td>
<td>15.76±2.96 (21.56-9.97)</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>8.66</td>
<td>4.43 x-3.08</td>
<td>6.67±0.74 (8.12-5.22)</td>
<td>1.30</td>
<td>12.99±2.50 (17.89-8.10)</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>5.70</td>
<td>4.12 x-2.25</td>
<td>5.75±0.74 (7.21-4.30)</td>
<td>1.51</td>
<td>11.78±2.23 (16.16-7.40)</td>
<td>1.48</td>
</tr>
</tbody>
</table>

(*Data reported in Bhan et al. 2013b)*

SE: Standard Error; UL: Upper Fiducial Limit; LL: Lower Fiducial Limit
Table -2 Lethal concentrations, LC$_{50}$ and LC$_{90}$ of different encapsulated nanoformulations of *A. flavus* and *C. reflexa* against An. *Stephensi*.

<table>
<thead>
<tr>
<th>Amount of pesticide used in Encapsulation (%)</th>
<th>Exposure period (hours)</th>
<th>Chi-Square</th>
<th>Regression equation</th>
<th>LC$_{50}$±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (with respect of exposure period)</th>
<th>LC$_{90}$±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (with respect of exposure period)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (%)</td>
<td>24</td>
<td>1.59</td>
<td>2.33x-1.19</td>
<td>46.13±8.79 (63.35-28.90)</td>
<td>4.39</td>
<td>163.94±51.87 (265.61-62.27)</td>
<td>6.45</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.57</td>
<td>1.51x+1.35</td>
<td>26.22±8.00 (41.91-10.54)</td>
<td>5.72</td>
<td>185.30±90.41 (362.49-8.10)</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.45</td>
<td>1.41x+1.87</td>
<td>16.30±6.51 (29.06-3.54)</td>
<td>6.58</td>
<td>131.39±62.19 (253.29-9.48)</td>
<td>4.87</td>
</tr>
<tr>
<td>2 (%)</td>
<td>24</td>
<td>3.27</td>
<td>1.53x+1.87</td>
<td>11.16±3.52 (18.05-4.27)</td>
<td>18.15</td>
<td>77.04±39.24 (153.95-0.12)</td>
<td>13.73</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3.13</td>
<td>1.47x+2.28</td>
<td>7.07±2.23 (11.44-2.69)</td>
<td>21.21</td>
<td>52.73±26.37 (104.42-1.03)</td>
<td>16.19</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.09</td>
<td>1.35x+3.29</td>
<td>1.83±0.61 (3.02-0.26)</td>
<td>58.59</td>
<td>16.38±8.22 (32.49-0.26)</td>
<td>39.02</td>
</tr>
<tr>
<td>4 (%)</td>
<td>24</td>
<td>2.63</td>
<td>2.08x-1.09</td>
<td>85.73±19.70 (124.35-47.12)</td>
<td>2.36</td>
<td>355.32±158.56 (666.10-44.53)</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.17</td>
<td>1.97x-0.56</td>
<td>67.01±15.24 (96.88-37.15)</td>
<td>2.24</td>
<td>300.19±132.11 (559.11-41.26)</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.91</td>
<td>1.92x-0.28</td>
<td>56.29±12.89 (81.56-31.01)</td>
<td>1.90</td>
<td>261.80±113.89 (485.03-38.57)</td>
<td>2.44</td>
</tr>
<tr>
<td>8 (%)</td>
<td>24</td>
<td>3.41</td>
<td>1.79x-0.91</td>
<td>202.62±53.57 (307.62-97.62)</td>
<td>1.00</td>
<td>1057.43±502.25 (2041.84-73.02)</td>
<td>1.00</td>
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<tr>
<td></td>
<td>48</td>
<td>3.07</td>
<td>1.69x-0.39</td>
<td>149.92±39.85 (228.02-71.81)</td>
<td>1.00</td>
<td>853.69±415.71 (1668.49-38.91)</td>
<td>1.00</td>
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<tr>
<td></td>
<td>72</td>
<td>1.07</td>
<td>1.66x-0.01</td>
<td>107.22±28.24 (162.57-51.87)</td>
<td>1.00</td>
<td>639.06±306.12 (1239.06-39.06)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

SE: Standard Error; UL: Upper Fiducial Limit; LL: Lower Fiducial Limit
Table -3 Lethal concentrations, LC$_{50}$ of different encapsulated nanoformulations of *A. flavus* and *C. reflexa* against *Cx. quinquefasciatus*.

<table>
<thead>
<tr>
<th>Amount of pesticide used in Encapsulation (%)</th>
<th>Exposure period (hours)</th>
<th>Chi-Square</th>
<th>Regression equation</th>
<th>LC$_{50}$±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (with respect of exposure period)</th>
<th>LC$_{90}$±SE (UL-LL) (mg/L)</th>
<th>Relative toxicity (with respect of exposure period)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(%)</td>
<td>24</td>
<td>3.16</td>
<td>2.27x-1.16</td>
<td>52.06±10.04 (71.74-32.38)</td>
<td>4.99 (191.27±4171.12 (317.86-64.69)</td>
<td>191.27±4171.12 (317.86-64.69)</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.69</td>
<td>2.30x-1.01</td>
<td>40.66±7.95 (56.23-25.08)</td>
<td>5.59 (146.23±45.07 (234.56-57.89)</td>
<td>146.23±45.07 (234.56-57.89)</td>
<td>7.24</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.32</td>
<td>2.11x-0.31</td>
<td>32.59±7.05 (46.41-18.78)</td>
<td>6.16 (131.63±42.82 (215.56-47.69)</td>
<td>131.63±42.82 (215.56-47.69)</td>
<td>6.15</td>
</tr>
<tr>
<td>2(%)</td>
<td>24</td>
<td>3.09</td>
<td>1.36x+2.10</td>
<td>13.37±4.99 (23.15-3.59)</td>
<td>19.43 (116.55±59.35 (232.88-0.21)</td>
<td>116.55±59.35 (232.88-0.21)</td>
<td>8.09</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.93</td>
<td>1.44x+2.27</td>
<td>7.73±2.86 (13.34-2.12)</td>
<td>29.42 (59.70±29.70 (117.91-1.49)</td>
<td>59.70±29.70 (117.91-1.49)</td>
<td>17.73</td>
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<td></td>
<td>72</td>
<td>1.49</td>
<td>1.83x+2.21</td>
<td>3.36±1.11 (5.55-1.18)</td>
<td>59.71 (16.88±8.60 (33.73-0.03)</td>
<td>16.88±8.60 (33.73-0.03)</td>
<td>48.10</td>
</tr>
<tr>
<td>4(%)</td>
<td>24</td>
<td>1.85</td>
<td>2.31x-2.89</td>
<td>259.79±55.11 (367.81-151.77)</td>
<td>1.00 (931.46±331.39 (1580.99-281.94)</td>
<td>931.46±331.39 (1580.99-281.94)</td>
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</tr>
<tr>
<td></td>
<td>48</td>
<td>1.22</td>
<td>2.25x-2.57</td>
<td>227.36±49.64 (324.65-130.06)</td>
<td>1.00 (841.35±298.81 (1427.02-255.68)</td>
<td>841.35±298.81 (1427.02-255.68)</td>
<td>1.26</td>
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<tr>
<td></td>
<td>72</td>
<td>1.78</td>
<td>2.12x-1.99</td>
<td>200.85±46.02 (291.06-110.65)</td>
<td>1.00 (809.74±305.65 (1408.82-210.66)</td>
<td>809.74±305.65 (1408.82-210.66)</td>
<td>1.00</td>
</tr>
<tr>
<td>8(%)</td>
<td>24</td>
<td>1.66</td>
<td>1.98x-1.60</td>
<td>213.03±48.62 (308.32-117.73)</td>
<td>1.22 (942.67±370.17 (1668.20-217.14)</td>
<td>942.67±370.17 (1668.20-217.14)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.78</td>
<td>1.68x-0.50</td>
<td>183.61±46.97 (275.67-91.54)</td>
<td>1.24 (1058.42±494.52 (2027.69-89.16)</td>
<td>1058.42±494.52 (2027.69-89.16)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.84</td>
<td>1.75x-0.56</td>
<td>147.77±37.05 (220.40-75.14)</td>
<td>1.36 (794.08±341.57 (1463.55-124.61)</td>
<td>794.08±341.57 (1463.55-124.61)</td>
<td>1.02</td>
</tr>
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</table>

SE: Standard Error; UL: Upper Fiducial Limit; LL: Lower Fiducial Limit
DISCUSSION

In recent years, biodegradable polymeric nanoparticles (NPs) have become most vital field of research owing to their small size and large surface area and because they exhibit unique properties not seen in bulk materials (Prasad et al. 2014)\(^{11}\). This property attracted considerable attention as potential pesticide delivery devices in view of their applications in the control release (CR) of pesticide, their ability to target particular organism. Thus, the major benefit of employing nanopesticides is the opportunity to enhance properties such as efficacy and specificity. However, application of nanoparticles in insect pest management and mosquito control is still at rudimentary stage.

Our study represents the work in the field of biodegradable polymeric nanoparticles used as pesticide delivery systems. The nanoencapsulation of pesticides mixture were prepared by melt-dispersion method, an easy and most economical method for preparing pesticide loaded nanoparticles (Peng et al. 2008)\(^{23}\). PEG has been selected in our work as a polymeric matrix as it possesses various outstanding properties including biodegradable, biocompatible, hydrophilicity and lack of toxicity (Yang et al. 2009)\(^{28}\). In addition, nano-encapsulation renders liquid form of pesticides into powdered form to prevent clumping and improve mixing and helps in occlusion of their odor (Soraf et al. 2007)\(^{29}\). Thus, nanoencapsulation of pesticides mixture can significantly enhance their pesticidal life, make them easily dispersible powders and also improves their specificity.

The LC\(_{50}\) of nanoparticles mixture (2\%) against anopheline and culicine larvae were 11.16, 7.07 and 1.83 mg/L and 13.37, 7.73 and 3.36 mg/L after 24, 48 and 72 hrs of exposure periods. The amount of pesticides used in nanoparticles mixture were much lesser than the non-encapsulated mixture. The LC\(_{50}\) values of non-encapsulated synergistic mixture were LC\(_{50}\) values 7.910, 6.368 and 4.317 mg/L against anopheline larvae and in case of culicine larvae LC\(_{50}\) values were 8.712, 6.671 and 5.755 mg/L after 24, 48 and 72 hrs respectively. Initially the rate of release of pesticide from CRF was much slower than their conventional forms. It was also noted that the toxicity of the encapsulated nanoparticles mixture gradually increased with increase in time as compared to conventional synergistic mixture. This observation is supported by the findings of Yang et al. (2009)\(^{28}\). The application of CRF regulates the supply of nanopesticides mixture at the required rate for mosquito control thus reducing the amount of pesticides introduced in the environment and protecting it from its deleterious effects. Kuzuma and Verhage (2006)\(^{30}\) found that the nanopesticides are highly toxic as a result, the required quantities of pesticide used could be reduced leading to reduction in pollution load, ultimately reducing input costs. Cao et al. (2005)\(^{31}\) reported that nanoformulation of pesticides is safer from an environmental point of view. Nano-scale formulations of new and existing pesticidal toxins offer a variety of novel properties, such as the controlled or targeted release of...
pesticides, e.g., in the alkaline nature of certain insects’ digestive systems, or under strict moisture and heat levels (ETC group 200432; FoE 200833).

Similar work has been observed for imidacloprid and isoproturon-controlled release formulations from alginate-bentonite-activated carbon formulations by Herrera et al. (2006)34 and Adak et al. (2012)35 for the development of controlled release formulations of imidacloprid employing novel nano-ranged amphiphilic polymers. Debnath et al. (2010)36 who tested entomotoxicity of silica nanoparticles against rice weevil, Sitophilus oryzae and its efficacy was compared with its bulk form. Anjali et al. (2010)37 has prepared water dispersible nanopermethrin using solvent evaporation and investigated its larvicidal property against Cx. quinquefasciatus. The results showed that the nanopermethrin was more potent in its larvicidal effect (0.117mg/l) than the bulk form of permethrin (0.715mg/L). Patel et al. (1990)38 compared the six different types and concentrations of alginate for encapsulation of Lagenidium giganteum against Cx. quinquefasciatus larvae and found that fungus within the lower concentration alginate is more effective. Paula et al. (2011)39 has found that cashew gum (CG) nanoparticles as protective coating of a natural larvicide extracted from Moringa oleifera seeds and evaluated its larval activity against Stegomya aegypti with 98±3 % mortality. Guan et al. (2008) directly evaluated the toxicity of nano-imidacloprid against the adult, Martianus dermestoides. The formulation and release kinetics of novel Imidacloprid nano-formulation in soyabean fields was studied by Guan et al. (2010)40. Recently, Rai and Ingle (2012)41 reviewed the applications of nanotechnology in controlling and management of pests in agriculture. Chitosan/Cashew gum microspheres were developed for encapsulation of DDVP (dimethyldivinyl chloride phosphate) aiming at larvae control by Paula et al. (2006)42.

The present work reveals that nanoencapsulated mixture requires lesser phytotoxics than their conventional mixture, eliminates the necessity of using volatile organic solvents in the formulation, and presents a much “greener” method for mosquito control. Thus, pollution load from encapsulated nanoformulations could be significantly reduced. It could serve as an environmental friendly and effective insecticide with selective toxicity to insect pests. In the tested target species, nanomodified pesticide caused a greater toxic response at lower concentration compared to their conventional counterparts. It can be concluded that encapsulated nanoformulations with 2% of mixture can be used as a safe and effectual alternative to mosquito control.

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