Larvicidal activity of Green Clean (GC) at various concentrations

Work done by,
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19th Feb., 2015

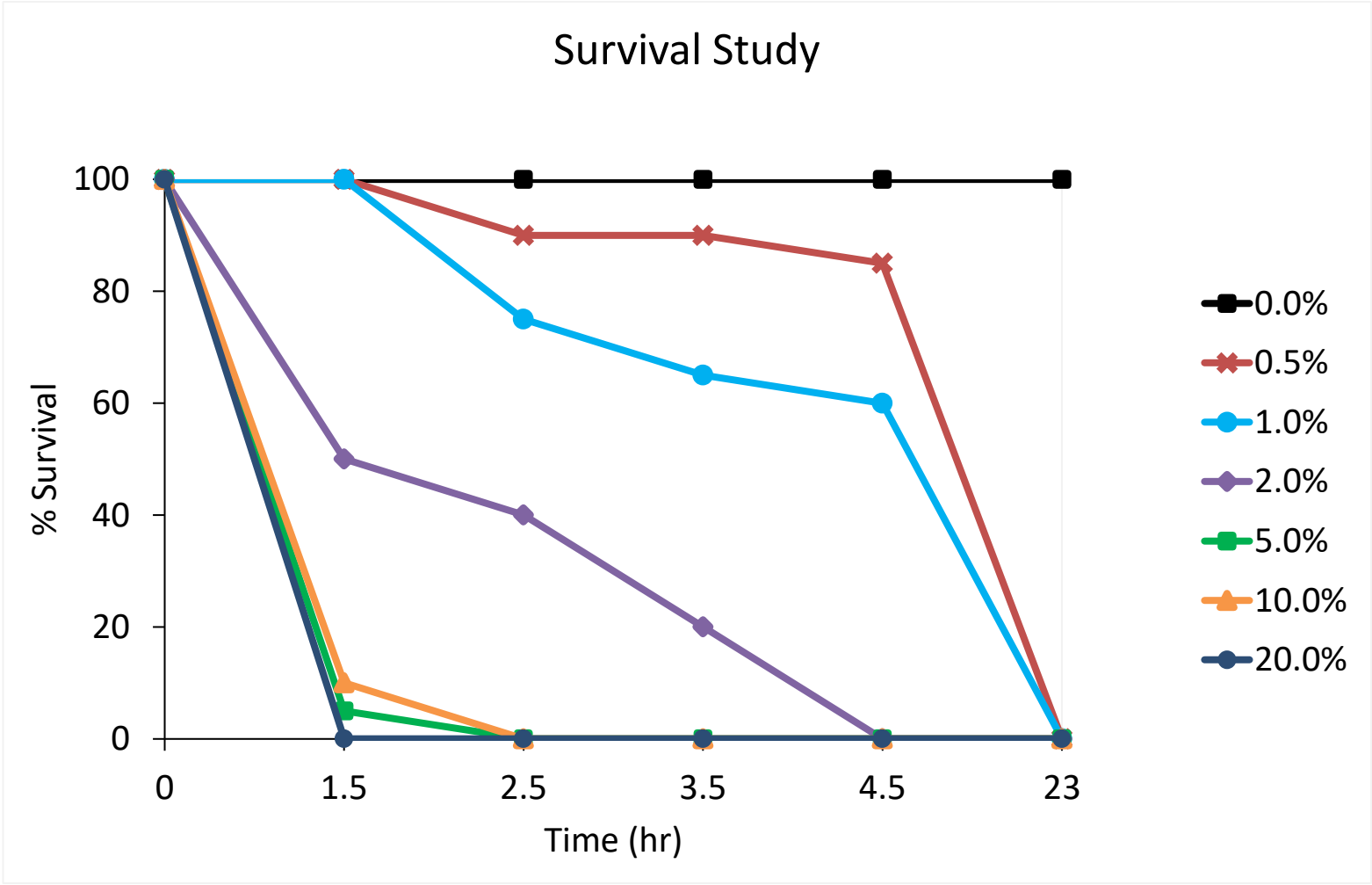
Conditions

- Temperature: 27^{o}C
- Humidity: 70-75%
- Experiment done in: 6 well plate
- Water volume: 10 ml/well
- Larval stage: 2nd instar
- No. of Larvae: 10/well
- % Dilution of GC in water (Final): 0, 0.5, 1, 2, 5, 10, 20%
- Replicates: 2
- Strain: *Anopheles stephensi*

Observations

- % movement (Direct Observation)

Time	0 hr		1.5 hr		2.5 hr		3.5 hr		4.5 hr		23 hr	
	% Movement		% Movement		% Movement		% Movement		% Movement		% Movement	
Dilution	Well 1	Well 2	Well 1	Well 2	Well 1	Well 2	Well 1	Well 2	Well 1	Well 2	Well 1	Well 2
0%	100	100	100	100	100	100	100	100	100	100	100	100
0.5%	100	100	100	100	90	90	90	90	90	80	0	0
1%	100	100	100	100	60	90	60	70	60	60	0	0
2%	100	100	50	50	50	30	20	20	0	0	0	0
5%	100	100	10	0	0	0	0	0	0	0	0	0
10%	100	100	10	10	0	0	0	0	0	0	0	0
20%	100	100	0	0	0	0	0	0	0	0	0	0


Survival Study
100
80
60
40
20
0
% Survival
0
1.5
2.5
3.5
4.5
23
Time (hr)
0.0%
0.5%
1.0%
2.0%
5.0%
10.0%
20.0%

Effect of GC on Larvae at 5 hr

0.0%

0.5%

1.0%

2.0%

5.0%

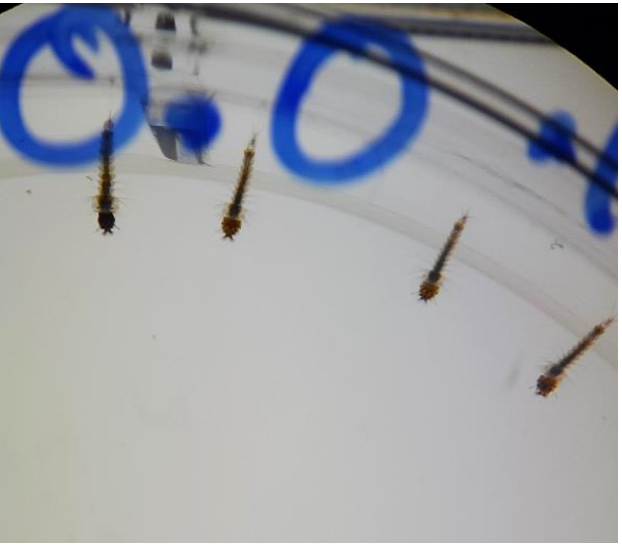
10.0%

Effect of GC on Larvae at 5 hr

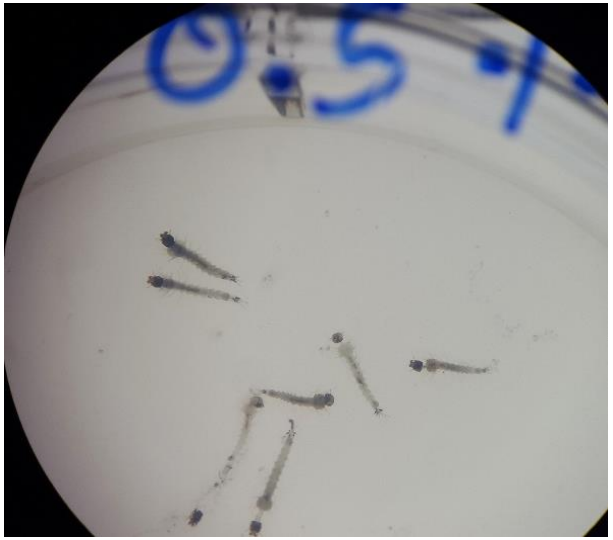
20.0%

Effect of GC on Larvae at 24 hr

0.0%



0.5%



1.0%

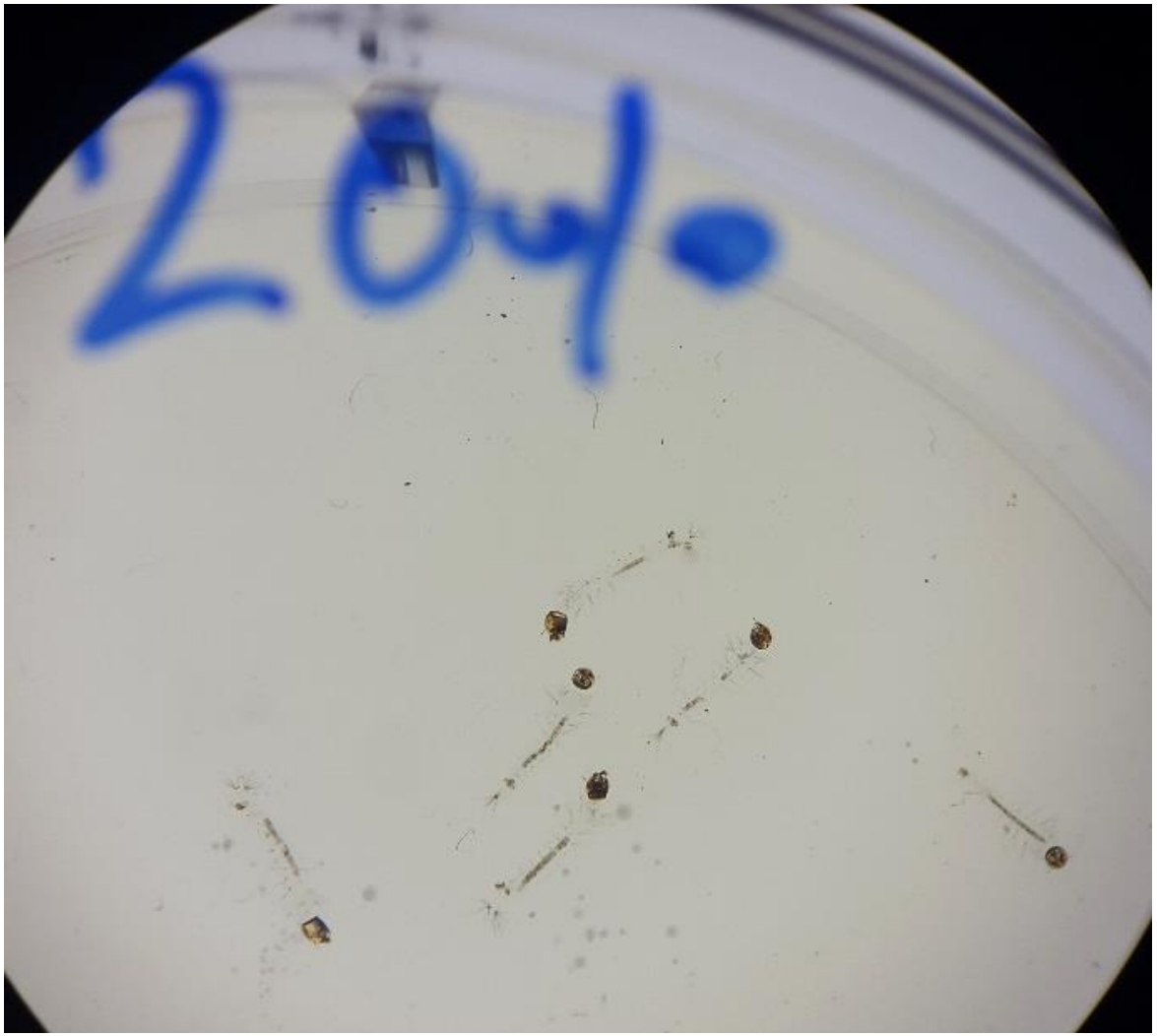
2.0%

5.0%

10.0%

Effect of GC on Larvae at 24 hr

20.0%



Progress Report [February, 2015 – January, 2016]

Section A: Project Details

- **Project Title:** Evaluation of the effect of Green Clean Product (GCP), derived from plants, on various developmental stages of Mosquitoes

- **Background:** Mosquitoes are the prime vectors for many life threatening diseases including malaria, dengue, chikungunya, yellow fever, encephalitis etc. around the globe. For long, people have been taking various measures to prevent the mosquito growth. The major preventive measures include the use of insecticides (mainly DTT) which has greatly reduced the mosquito burden. However, insecticide resistant strains of mosquitoes have again posed the threat of increasing the disease incidences. Therefore, there is an urgent need for alternative strategies which could prevent the mosquito growth and at the same time minimize the generation of the resistant strains. Latest development in nanotechnology seems to be highly promising in making compounds which could be used for such purpose. Preliminary study using "Green Clean Product (GCP)" has shown that mosquito growth can be prevented at different developmental stages. GCP is a nanoparticle formulation made from various plants extracts, and hence is eco-friendly and safe to human health. This research proposal intends to study in the systematic way the effect of GCP in controlling the mosquitoes growing under various conditions.

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- **Total costs:** Rs. 2,05,400.00

- **Duration:** 8 months* (February, 2015 – September, 2015)
  * Project has been extended upto March, 2016

- **Objectives of the Project:**
  - ✓ To test the effect of GCP in dose (conc.) dependent manner on the survival of Larvae and Pupa.
  - ✓ To determine the time required for killing the larvae and pupa.
  - ✓ To test the effect of GCP on prevention of hatching the eggs.
  - ✓ To find out the best possible methods of applying the product to prevent the mosquito growth.

Section B: Technical Progress Report

⇨ **Obj. 1: To test the effect of GCP in dose (conc.) dependent manner on the survival of larvae and pupa.**

Based on WHO guidelines [1], we tested the larvicidal effect of GCP on wild type larvae of *Culex*, *Aedes* and *Anopheles* species of mosquito. Approximately 50 larvae were taken in 200 ml of RO (Reverse Osmosis) water. We added GCP in various volume to achieve the final concentrations i.e. 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05%.

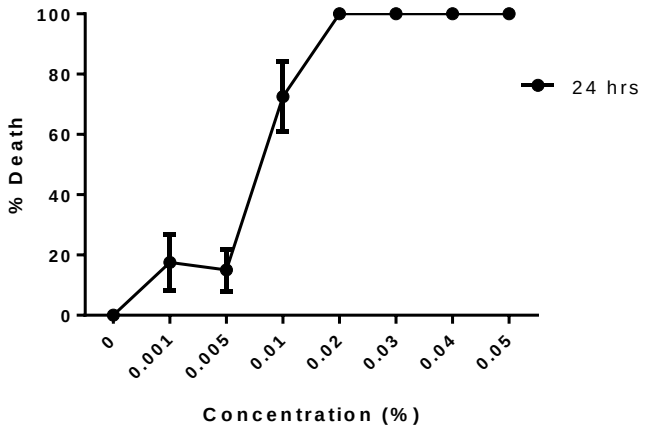


Figure 1. **Larvicidal effect GCP on mosquito larvae at various concentration.** Wild type larvae of *Culex*, *Aedes* and *Anopheles* were taken, particularly which are in 3rd and 4th instar stage of development (50/vessel). Environmental conditions (Temperature: 26-27°C; Humidity: 70-75%, optimal for the growth of mosquito larvae) was maintained as per the requirement. Larvicidal effect of GCP at 24 hr post treatment was evaluated with various concentrations in four replicates. Data are presented as the means ± SD and representative of one of the three independent experiments.

We found that 0.02% is the minimum concentration which has shown 100% larval killing (Figure 1). During these experiments, standard larval food was not provided. However, when the same experiments were performed with the inclusion of larval food, we found decrease in death rate of the larvae from 100% to 67.5% at 0.02% GCP concentration, whereas 100% mortality was seen at 0.03% GCP concentration in all the larval spp. (Figure 2).

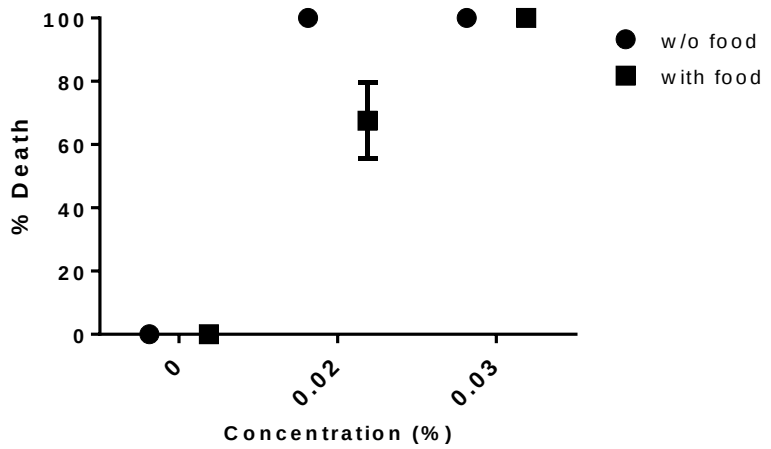


Figure 2. **Larvicidal effect GCP on mosquito larvae with and without inclusion of larval food.** Wild type larvae of *Culex*, *Aedes* and Environmental conditions (Temperature: 26-27^{o}C; Humidity: 70-75%, optimal for the growth of mosquito larvae) was maintained as per the requirement. One set of test vessels with the described concentrations was kept without supplementing larval food (Dark circles), whereas in another set larval food was supplemented (Dark squares). Larvicidal effect of GCP at 24 hr post treatment was evaluated with various concentrations in duplicates. Data are presented as the means ± SD and representative of one of the two independent experiments.

Further, we tested the lethal activity of GCP on the pupal stage of *Culex, Aedes* and *Anopheles* spp. of mosquitoes. We found that 0.03% is the minimum concentration which has shown 100% pupal killing (Figure 3).

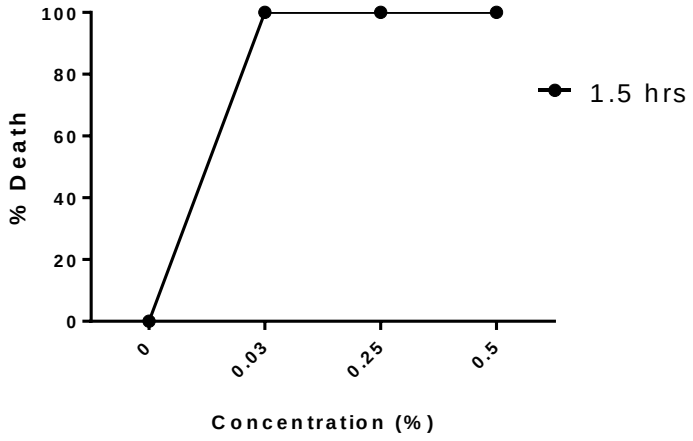
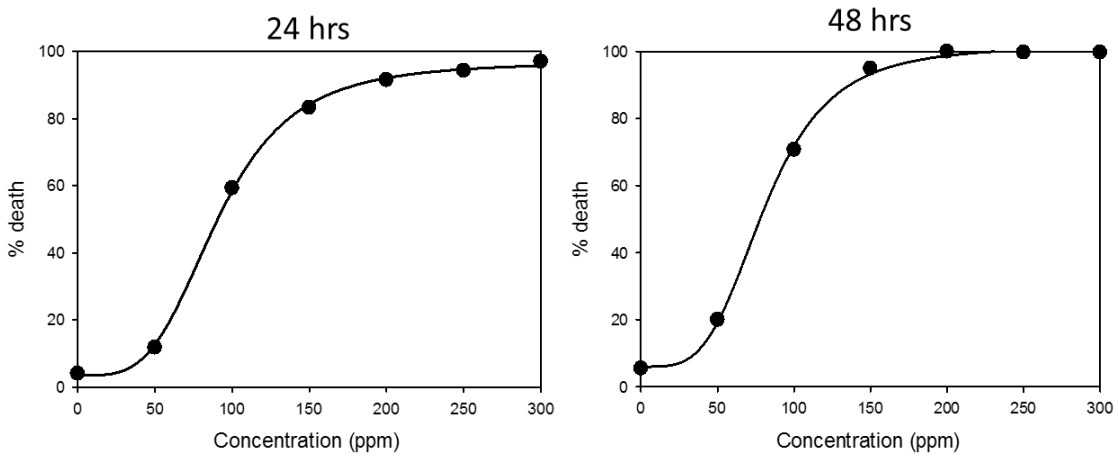


Figure 3. **Lethal activity of GCP on pupa.** Wild type pupa of *Culex* and *Aedes* were taken (50/vessel). Environmental conditions (Temperature: 26-27^{o}C; Humidity: 70-75%, optimal for the growth of mosquito pupa) was maintained as per the requirement. Lethal effect of GCP was tested in 4 replicates with various concentrations for 1.5 hrs of time duration. Data are presented as the means ± SD and representative of one of the five independent experiments.

⇨ **<u>Obj. 2</u>: To determine the time required for killing the larvae.**

According to WHO guidelines for laboratory and field testing, larvicides should be effective for their larvicidal activity in 24 -48 hrs. Hence, we tested the larvicidal activity of GCP post 24 hrs or 48 hrs treatment in concentration (in ppm) dependent manner.



<u>Figure 4</u>. Larvicidal effect GCP on larval stage at 24 hrs or 48 hrs post treatment. Wild type larvae of *Culex*, *Aedes* and *Anopheles* were taken, particularly which are in 3rd and 4th instar stage of development. Environmental conditions (Temperature: 26-27^{o}C; Humidity: 70-75%) have been maintained which are optimal for the growth of mosquito larvae. Larvicidal effect of GCP at 24 hr and 48 hr post treatment was evaluated with various concentrations in eight replicates. Here, concentrations are shown in the form of ppm where 1% equals to 10,000 ppm concentration.

We found increased larval death even when kept for 48 hrs. The Lethal concentration 50 (LC50) were 90.77 ppm and 81.11 ppm for 24 hrs and 48 hrs, respectively.

⇨ **<u>Obj. 3</u>: To test the effect of GCP on prevention of hatching the eggs.**

To determine whether GCP can prevent the hatching of the eggs, we first collected eggs from *Anopheles spp.* of mosquitoes in OVI trap plastic bowls. We added larval food in very low quantity. After that we added GCP with final concentration of 0.02% (200ppm). We kept bowl in optimum environmental conditions for 24 -48 hrs to induce the hatching process. We found no effect of GCP on the hatching process at 0.02% concentration compared to control. At present, we are testing with the higher concentrations of GCP.

⇨ **<u>Obj. 4</u>: To find out the best possible methods of applying the product to prevent the mosquito growth.**

The backyard area of the Animal House and the Herbal Garden at Nirma University have been spotted for small scale field trials for GCP as they are well suited as a natural habitat for larval development. For the application of GCP in the field trails, we adopted two methods for application of GCP on mosquito larvae found in natural habitat i.e., 1) Pouring; 2) Spraying. We applied GCP in plastic containers, flower pots etc., in which we found the larvae mostly of *Aedes* and *Anopheles spp.* mosquitoes.

1) In pouring method, we dropwise added GCP directly from the stock in such a way so that the final concentration would be approximately ranging from 0.03%-0.06%. With this method of application, we found 0.04 -0.06% concentrations are more effective for 100% mortality. This method of application has shown higher efficacy particularly in the conditions where the depth of water is more than 5-10 cm.
2) In spraying method, we used 5,10 and 20% solution of GCP as a stock solution. We sprayed the stated % solutions using regular spraying bottle (500 ml capacity) in the field as well as in larvae containing plastic trays in laboratory. But initially we determined the volume of GCP solution ejected in each spray. Accordingly, we sprayed the solution to get the final desired concentration (Range 0.03 – 0.06%). We found 0.05 and 0.06% concentrations have shown 100% mortality. This method of applications has shown higher efficacy only when the depth of water is ≤5-10 cm.

Summary and Conclusions of the progress made so far:

We tested the Green Clean Product (GCP) for its larvicidal activity on wild type larvae (*Anopheles*, *Culex* & *Aedes* spp.), collected from the field. GCP has shown its highest efficacy at concentration as low as 300 ppm (0.03%) for 100% larval mortality within 24-48 hrs in the laboratory experiments. It has shown lethal effect on pupal stage of mosquito development at the concentration of 300 ppm (0.03%) in 1.5 hrs. We tested GCP in small scale field trials where we found 0.05% is the minimum effective concentration for killing the larvae within 24-48 hrs in all possible way of application. GCP can be used for large scale field trials.

References:

1. WHO. Dept. of Communicable Disease Prevention CaE. Guidelines for laboratory and field testing of mosquito larvicides. **2005**.

ANNEX 1: Dilutions and Concentrations

%	ppm (parts per million)	GCP Stock volume/100ml of water (µl)
0	0	0
0.001	10	1
0.005	50	5
0.01	100	10
0.02	200	20
0.03	300	30
0.04	400	40
0.05	500	50
0.06	600	60

ANNEX 2: Field Application (Effective Concentration: 0.05%)

Water volume (litre)	GCP volume (ml)
1	0.5
5	2.5
10	5
50	25
100	50
200	100
500	250
1000	500

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Nirma University

09th September, 2015

Objectives

a) To test the effect of GCP in dose (conc.) dependent manner on the survival of Larvae and Pupa.

b) To determine the time required for killing the larvae and pupa.

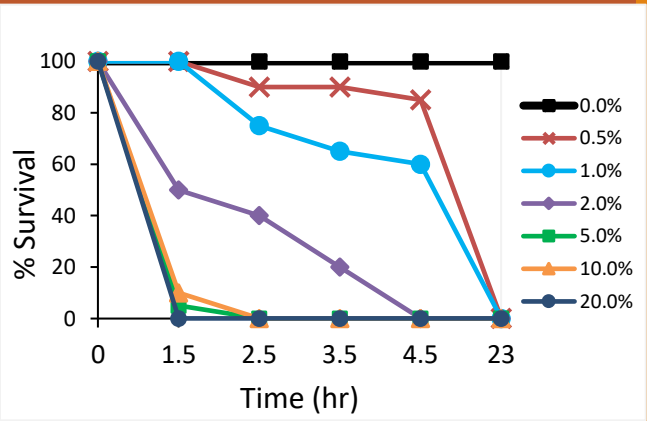
c) To test the effect of GCP on prevention of hatching the eggs.

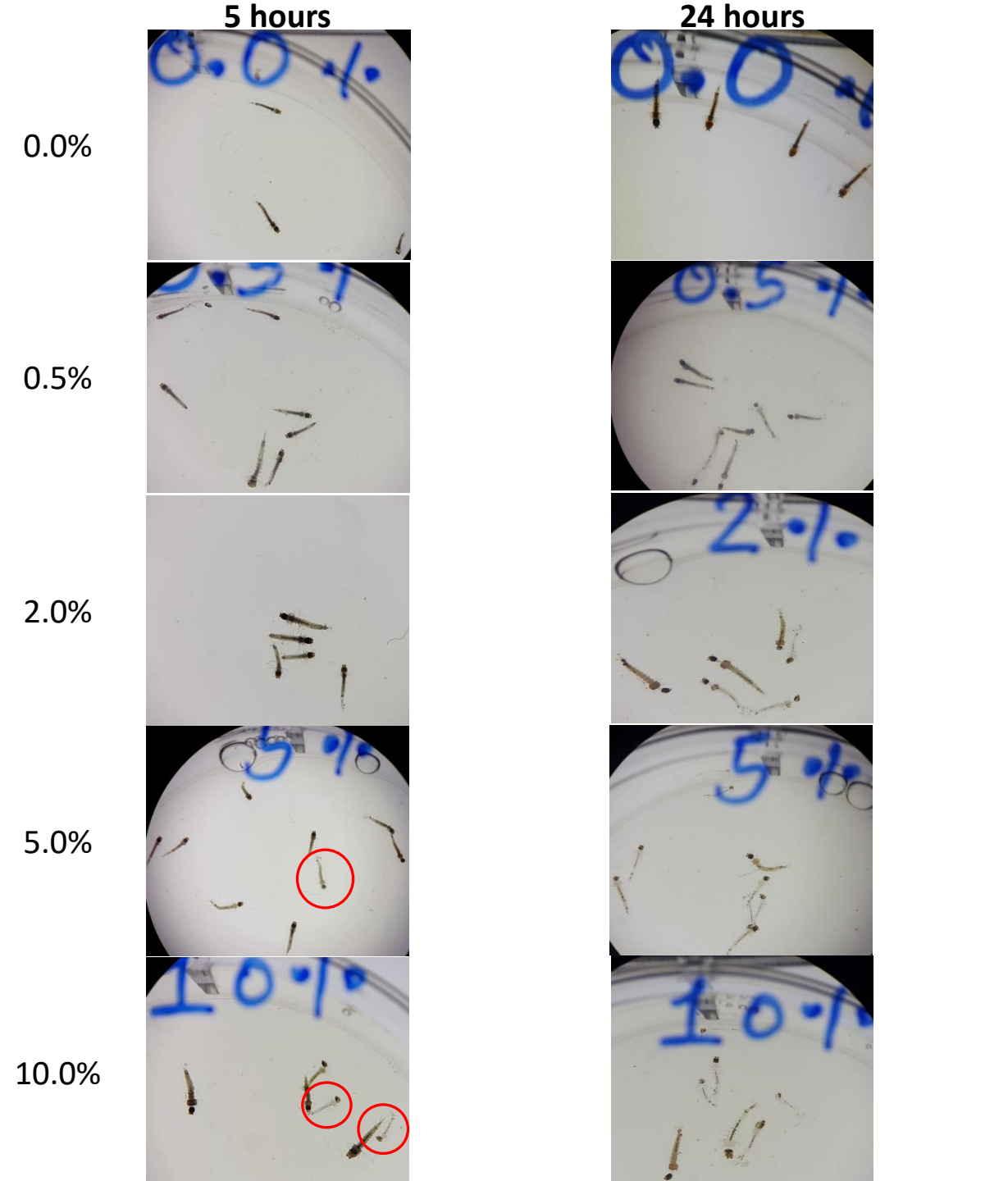
d) To find out the best possible methods of applying the product to prevent the mosquito growth.

Work Done so far

Conditions

- Temperature: 27°C
- Humidity: 70-75%
- Experiment done in: 6 well plate
- Water volume: 10 ml/well
- Larval stage: 2nd instar
- No. of Larvae: 10/well
- % Dilution of GCP in water (Final): 0, 0.5, 1, 2, 5, 10, 20%
- Replicates: 2
- Strain: ***Anopheles stephensi***

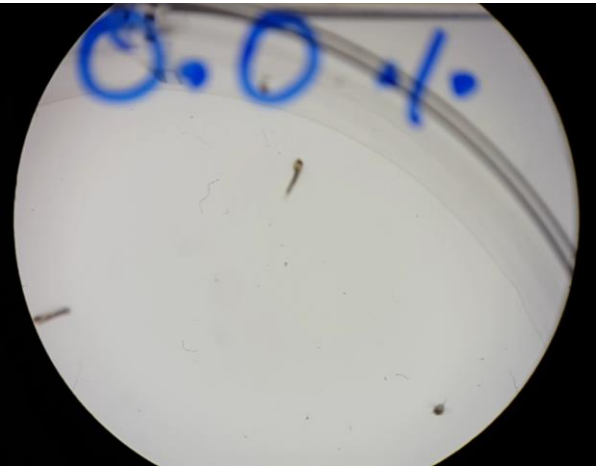




Microscopic Observations (*Anopheles stephensi* larvae)

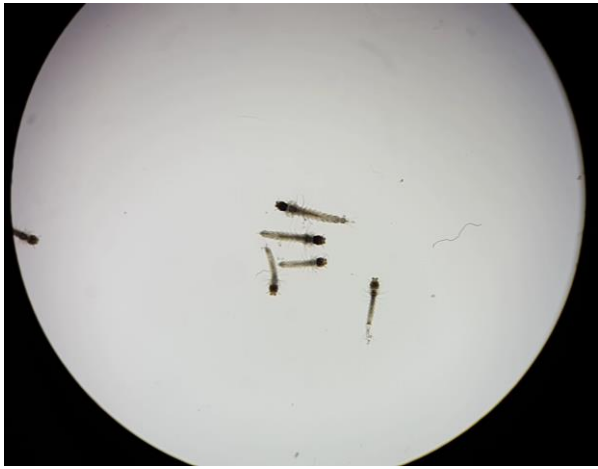
5 hours

0.0%



0.5%

2.0%



24 hours

0.0%

0.5%

2.0%

Direct Observations (Wild type larvae)

Conditions

- Temperature: 27°C
- Humidity: 70-75%
- Experiment done in: Plastic vessel
- Water volume: 200 ml/ vessel
- No. of Larvae: 50/ vessel
- Larval stage: 3rd & 4th instar
- Strain: ***Culex* & *Aedes***

0 hour

22 hours

Direct Observations (Wild type Pupa)

Conditions

- Temperature: 27°C
- Humidity: 70-75%
- Experiment done in: Plastic vessel
- Strain: ***Culex* & *Aedes***

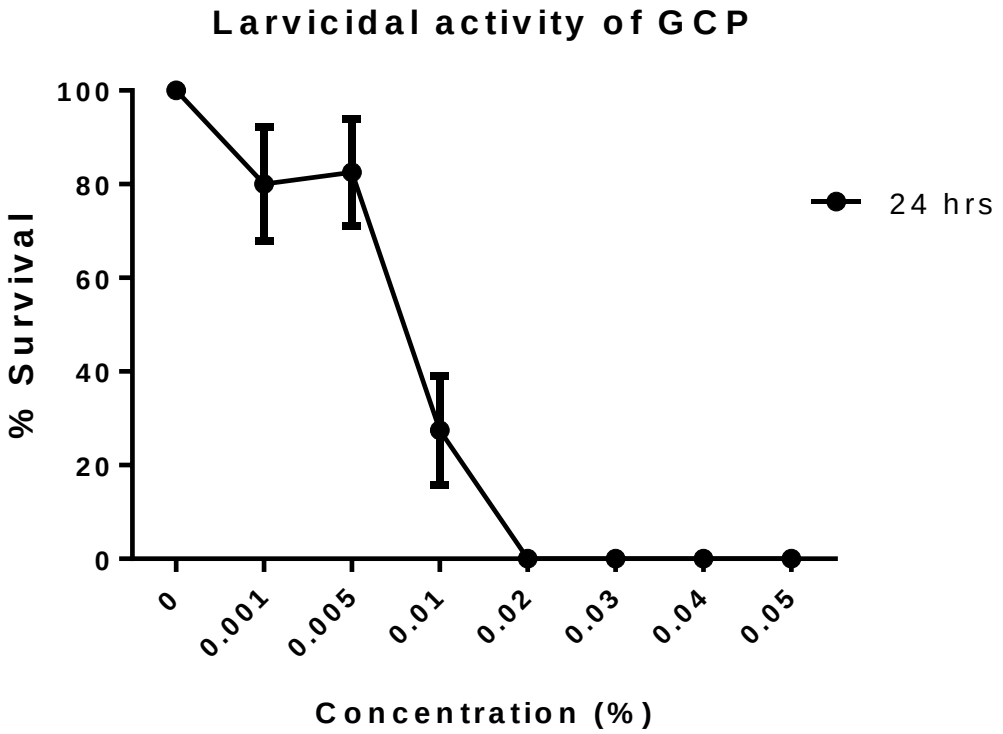
0 hour

1.0 hour

Minimum effective concentration

Conditions

- Temperature: 27°C
- Humidity: 70-75%
- Experiment done in: Plastic vessel
- Water volume: 200 ml/vessel
- Larval stage: 3rd & 4th instar
- No. of Larvae: 25 – 30/ vessel
- % Dilution of GCP in water (Final): 0, 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05%
- Replicates: 4
- Strain: ***Culex & Aedes***



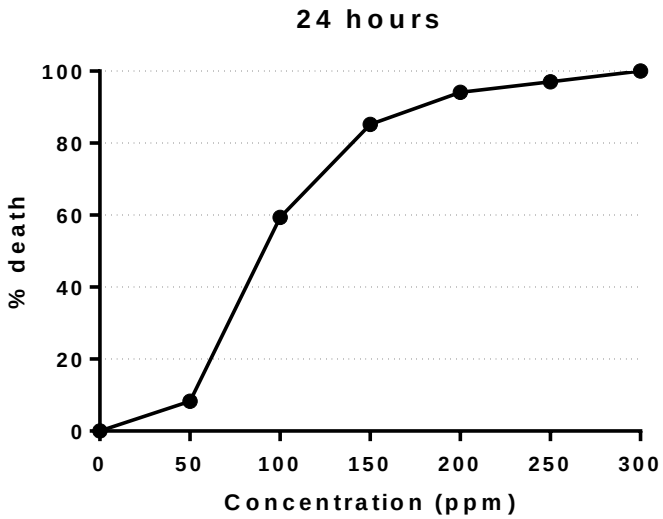
Time vs concentration graph

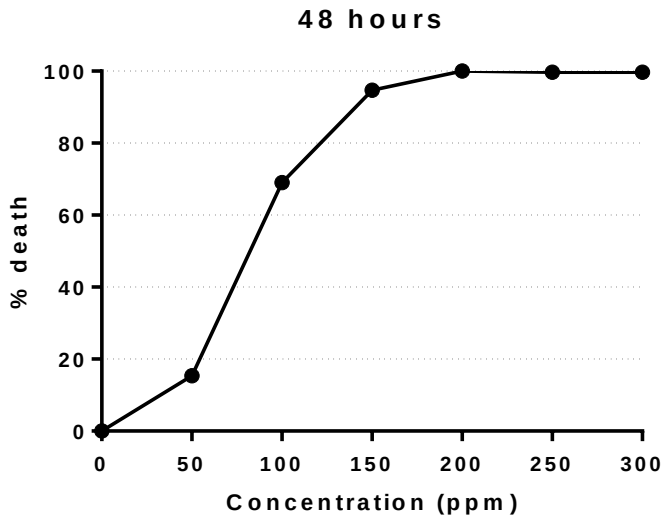
Material: Green Clean product | Formulation: in water | Temp.: 26-27 °C | Lighting: 12D/12N

Species: Culex & Anopheles | Larval instar: 3rd & 4th | Larvae/vessel: 50

Water: RO | Volume of water: 200ml | Food: -

	No. of **Dead Larvae** at various conc. (ppm) post exposure (hr.)													
	24 hr							**48 hr**						
Replicate	**0**	**50**	**100**	**150**	**200**	**250**	**300**	**0**	**50**	**100**	**150**	**200**	**250**	**300**
1	0	5	35	49	37	42	47	0	5	39	50	50	50	50
2	0	4	47	50	39	48	49	0	7	47	50	50	50	50
3	0	5	31	40	45	46	46	1	8	34	50	50	50	50
4	1	1	26	44	45	46	47	1	2	29	47	50	50	50
5	2	7	19	16	50	50	50	2	12	38	39	50	50	50
6	2	6	26	39	50	48	50	7	12	32	45	50	50	50
7	5	7	31	48	50	47	50	5	10	39	50	50	49	50
8	6	12	22	47	50	50	49	6	24	25	49	50	50	49
Average	2	5.875	29.625	41.625	45.75	47.125	48.5	2.75	10	35.375	47.5	50	49.875	49.875
% death	4	11.75	59.25	83.25	91.5	94.25	97	5.5	20	70.75	95	100	99.75	99.75





Conditions [with or without Food]

Conditions

- Temperature: 27°C
- Humidity: 70-75%
- Experiment done in: Plastic vessel
- Water volume: 200 ml/vessel
- Larval stage: 3rd & 4th instar
- No. of Larvae: 100/ vessel
- % Dilution of GCP in water (Final): 0, 0.02, 0.03%
- Replicates: 2
- Strain: ***Culex & Aedes***