Chlorophyll derivative activated with sunlight for malaria vector control: a small-scale field study

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Research Article

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Abstract

Background

Malaria is a life-threatening infectious disease transmitted through the bite of the *Anopheles* mosquito; hence, it could be prevented by a proper vector control. To date, this could be achieved by controlling adult mosquitoes using synthetic chemicals such as DDT for indoor residual spraying (IRS) and pyrethroid-treated bed nets. These approaches possess potential toxicities; therefore, a new ecologically safe technology for vector control was developed in this study.

Methods

Small-scale field studies were performed in the swamp with anopheline larvae from different sub-saharan countries, such as Sudan, Uganda and Ethiopia. Photodynamic control of anopheles larvae was employed using a chlorophyll derivative, pheophorbide-a (Ph-a) as a photosensitizer and sunlight as a light source. This could interrupt the life cycle of the *Anopheles* mosquitoes from the larval stage, which induces the interruption of the malaria disease cycle.

Results

Ph-a accumulates in the larval body and upon sunlight exposure, it induces oxidative stress, which causes 85 to 100% larval death 24 hours after treatment with Ph-a. This photosensitizer's effect persisted up to 21 days in the new generations in the same breeding site (residual effect). It is a target selective formula that has shown no effect on the other beneficial organisms in the breeding site.

Conclusions

This technique was found to be both effective and highly selective. It achieved a high mortality rate of mosquito larvae, while maintaining the highest levels of human safety and environmental friendliness.

Background

Malaria is a mosquito-borne and life-threatening infectious disease that begins with a bite from a female mosquito carrying the Plasmodium parasite [1]. According to the World Health Organization (WHO), each year, more than 400,000 people die of malaria. Almost 90% of these cases occur in the sub-Saharan African countries. An estimated two third of deaths are among children under the age of five [2] .

Many attempts have been made to control malaria by reducing human-vector contact or by disrupting the life cycle of female mosquitoes so that they do not survive long enough to transmit the parasite. Over the years, the ecological and epidemiological figures of malaria and the accessibility of strategies to combat

it have changed dramatically, making it essential to put new approaches to control this epidemic phenomenon. It has been reported that reducing malaria transmission is more useful and cost effective than managing infected cases. In Africa, it is not only the financial burden that matters, but also the developed resistance to conventional treatment strategies. For example, a resistance to chloroquinone and sulfadoxine-pyremethamine has increased, making it more challenging to manage malaria cases. Furthermore, up-to-date, none of the vaccine candidates developed have shown long-lasting benefits at a population level [3].

There are different strategies currently employed for the control of malaria in the epidemic countries, namely:

1. **Environmental control:** It is considered a long-term strategy through eradication of vector breeding areas, changing natural habitats or improving the human habitation to reduce the abundance of a target vector.
2. **Chemical control:** This includes residual spraying or larviciding and space spraying. This type of control is considered of relatively low cost with promising effective results.
3. **Biological control:** This is mainly based on eradication of the targeted mosquitoes through the utilization of selective biological toxins and natural enemies to achieve effective vector management. This type of control is usually more practically applicable with easily identifiable breeding places.

Genetically modified mosquitoes are one of the biological control methods of adult mosquitoes, releasing the mosquitoes adult that carry a modified lethal gene or sterile males to compete with normal ones [4,5] . In the sequence of this issue, this work introduces one more method of control called photodynamic vector control.

Photochemical process is a new emerging approach that was inspected at the laboratory level for the control of several types of insect populations [6-10]. The compounds of plant origin, named phototoxins or photosensitizers, have been isolated, examined and studied for combating a wide range of pests, including insects, fungi and weeds. Chlorophyll derivatives are among the main classes of phototoxins recently studied as photoparasiticides [11,12]. Our research group has reported the use of porphyrin-based compounds as efficient photosensitizing agents for potentially controlling *Culex pipiens* larvae, as well as other pests and parasites, which may be applied for several medical and veterinary applications [13-17].

In the present work, we aim at implementing a safe method in controlling malaria vector at the larval stage using photodynamic modality. Our goal is to interrupt the life cycle of the malaria vector by causing death of the *An. gambiae* larvae using a safe and cost-effective chlorophyll derivative as a photosensitizer.

Results

The confocal laser scanning microscope images and spectra showed the accumulation of Ph-a in the tissues of *Anopheles* sp. larvae in natural breeding sites. A part of the larva mid-gut and the transverse section of the same position showed the accumulated (red fluorescence) Ph-a in the alimentary canal (Fig. 1).

In Figs. 2 and 3, the arbitrary estimation of the fluorescence spectra of different larva body parts (fore-gut, mid-gut and hind-gut) showed an unfixed pattern of Ph-a accumulation as a function of incubation time. In Fig. 2, the relative intensity of chlorophyll in Anopheles larva tissue showed noticeable accumulation in different sites other than alimentary canal if it is compared with the background of CLSM image.

In Fig. 3, the fluorescence spectrum of Ph-a inside the alimentary canal showed the highest peak at 628 nm, but in the case of the fluorescence spectrum of Ph-a in the transverse section of larva at mid-gut (Fig. 4), the highest peak was 659 nm.

The field trials results are represented in Figs. 5, 6 and 7, which conclude the field activity in Sudan, Uganda and Ethiopia, respectively. They show the effect of different Ph-a concentrations on the mosquito larvae of genus *Anopheles* when exposed to sunlight under real environmental conditions. The results were monitored after 24 h from the onset of sunlight exposure. It was observed that 85%-100% *An. gambiae*, 77.6-100% *An. funestus* and 71-100% *An. arabiensis* larval mortalities were obtained as a function of the different Ph-a concentrations (0.06 mg/L up to 60 mg/L) (Figs. 5, 6 and 7, respectively). The effect of Ph-a with concentrations up to 100 mg/L was also tested on organisms that cohabit the same larvae breeding sites such as tadpole, *Gambusia* sp., and *Lymnea* snails, and were found not to be affected. Its effect was assessed after 1 day up to 7 days on those non-target organisms, and the results revealed a 100% survival rate. Both the control light group and the control dark group showed only maximum mortality of 2% in all experiments.

Discussion

The observations of the *Anopheles* larvae treated with Ph-a in their natural breeding sites by confocal laser scanning microscopy (CLSM) revealed the extent of Ph-a accumulation in the larva tissues as a function of larvae feeding. The time of Ph-a bleaching varied in the larval alimentary canal parts (Fig. 2 shows the Ph-a level in front and hind parts of the larval hind-gut). The dynamics of Ph-a accumulation and diminishing may modulate the photosensitization efficiency, as well as the rate of larval survival due to Ph-a aggregation at a high accumulation rate in the dark. This may affect the rate of production of reactive oxygen species (ROS) as a result of the photochemical reaction. The different characteristics of different mosquito breeding sites may alter the feeding behavior of mosquito larvae on Ph-a. The mosquito larvae of the breeding sites in less suspended organic materials have a high accumulation rate of Ph-a and vice versa. In both cases, we found that the percentage of mortality of mosquito larvae is still high in spite of turbid water.

It is worth mentioning that the field parameters should be taken into consideration before starting the scientific experiment. Focus should be done on monitoring the environmental conditions and the

awareness of the population in order to assess the efficacy, safety and community acceptability of utilizing new larvicides.

The results showed a promising success in eliminating vectors in a short period of time by disturbing the mosquito's life cycle without new generation formation or reinfestation. In Sudan, most of the infected mosquito breeding sites were agricultural activity of water accumulation ponds. These are temporary infected sites that maintain the seasonal supply of mosquitoes in the surrounding areas. Most mosquito-infected sites have beneficial living organisms such as tadpoles, water insects and aquatic snails. All these organisms were taken into consideration in this study. No lethal effect of Ph-a on these organisms up to concentration of 100 mg/L was observed during and after the experiments. This means that this naturally extracted Ph-a is a target selective formula that has shown no effect on the other beneficial microorganisms in the same breading site, assuring that this compound is an ecosystem friendly larvicide.

It was observed from the results of the field study on the three different species of *Anopheles* that the most sensitive one is *An. gambiae* (Fig. 6). It may be attributed to the clearness of the experimental mosquito-infected sites in Sudan that permitted more sunlight penetration through the water body. This is in agreement with our previous semi-field study in which less turbidity treated containers had higher photodynamic efficiency on mosquito larvae [19]. *An. funestus* and *An. Arabiensis,* which were tested against Ph-a in Uganda and Ethiopia, respectively, were still highly affected by experimental conditions at the same Ph-a concentrations. This may be due to the better quality of light in both Uganda and Ethiopia, as they are close to the equatorial plane and have less sunlight angle of incidence. This increases the efficiency of light penetration through the turbid water body.

No observable toxic effects were noticed against the non-target organisms that usually cohabit with mosquito larvae subjected to trial in water treated with Ph-a.

Methods

Chlorophyll derivative (pheophorbide-a) was obtained from INRAD Incorporation. Its purification (95% TC) was done using HPLC. The laboratory and field experiments were designed according to the WHO guideline.

Field investigations: The Anopheline breading sites (Blue Nile region in Sudan, Entebbe city in Uganda and Sidama zone in Ethiopia) were chosen and classified for this study according to several criteria including their nature (temporal or permanent), environmental characteristics of their close proximity, the physical and biological variables, and the weather conditions (Table 1). The breeding sites were chosen to be nearly similar in larval density and cohabitation organisms. The larval mortalities were assessed according to the average number of living larvae of four collecting spoons before and after treatment. The 3^{rd} and 4^{th} instars larvae were only considered in this work. Samples of mosquito larvae were

collected from treatment sites and were transferred to the laboratory to identify their species according to the anopheline identification keys [18,19].

Each experiment includes three replicates of treated breeding sites (T), light control breeding sites (LC) (breeding sites without chlorophyll treatment) and dark control breeding site (DC) (chlorophyll treated breeding sites without sunlight exposure, covered with a thin layer of black metal sheet hung on long sticks to avoid direct sunlight exposure and allowing air passage from the four directions). Each replicate of T, LC and DC includes three sites. 21 sites were used in each of the three countries. The required concentration of Ph-a at the treated site was prepared by dusting the appropriate amount of the Ph-a at the site to be treated according to the predetermined water surface area at the given site using hand-held duster (Prapo Poulos, Greece). Treated swamps were exposed to direct sunlight all day under the actual environmental conditions. Mosquito larvae were counted before and after treatment by taking aliquots from different positions of the experimental breeding site using a 250 ml spoon (3 spoons from 3 sides and one spoon from the center). The average number of living larvae from the four spoons of all site sides and center were counted for each breading site and the average number of living larvae was counted before and after treatment for all sites. The counted living larvae were sent back to their sites.

The percentage reduction in larval and pupa densities on post-treatment days was estimated for each site using Mulla's formula [20].

The experiments were repeated three times in the dry season. Each time, it was performed in different breeding sites having the same selection criteria.

Experiment on non-target organisms

Non-target specimens including larvivorous *Gambusia,* tadpoles, water spiders and *Lymnae* snails were collected. The organisms were kept for 24 h to acclimatize to the environment before being dosed with chlorophyll derivative. Three clean large basins of 54 cm diameter were provided and filled with 100 L natural pond water. The required amounts of the Ph-a were measured and the basins were dosed at the onset of the experiment. The date and time of the day were noted. Observations and bio-assays were conducted every 24 h for 3 days and on the 7^{th} day to check for acute and residual toxicity and mortality.

The sunlight fluence rate was measured with the Eldonet dosimeter (REAL TIME Co., Germany), which measures the average of UVA, UVB and visible light irradiances of sunlight during the exposure period.

Laboratory investigations

Anopheles larvae whole body and transverse sections preparation

The field-treated *Anopheles* larvae were fixed using paraformaldehyde. Also, no pigment was used during the preparation. In these experiments, pheophorbide-a (Ph-a) was used as a treatment agent, as well as a self-marker for distribution inside the tissue of *Anopheles* larvae. The specimens were put in small cryomolds with OCTTM compound (Tissue Tek embedding medium). All were kept inside the cryostats

until cutting. They were cut to a thickness of 13-24 µm using Jung Friocut 2800 N. The sections of specimens were mounted on Super-frost Plus microscope slides. Then, all slides were directly checked using Confocal Laser Scanning Microscopy (CLSM) (Carl Zeiss, Germany).

In addition, other samples required mounting the whole larva on the microscopic slide. In this preparation, the larva of *Anopheles* was transferred from paraformaldehyde to the microscope slide after little washing with alcohol for a few seconds. These specimens were mounted using mounting medium (Aquatex).

CLSM measurements

To acquire the best CLSM image using the LSM 410 inverted microscope, the optical path of the excitation laser beam and the fluorescence emission should be adjusted. Ph-a in these experiments can be excited using an argon laser beam (488 nm) from the LSM. The first dichroitic beam splitter (DBS1) was adjusted to a position of 510 nm. This position permits the argon laser beam (488 nm) to pass to the specimens and allows the passage of fluorescence emission beam from the specimen to the detector. The second dichroitic beam splitter was adjusted to the position of 560 nm to allow the wavelengths above 560 nm only to pass to the detector.

Data analysis

Mulla's formula

In this formula, percentage control or reduction (R) is calculated as:

$$\%R=100-[(C1/T1)x(T2/C2)]\times100$$

where C_1 = pretreatment measure of target species abundance in unsprayed control area, C_2 = post treatment unsprayed control, T_1 = pretreatment sprayed area, and T_2 = post treatment sprayed area. This formula is based on several basic assumptions:

(a) Counts in individual traps are independent measures of relative abundance.

(b) Ratio of abundance in traps in the control and treated areas is consistent over time.

(c) Changes in this ratio are due to treatment effects.

Data were assayed by analysis of variance (ANOVA), with the means of separation using Duncan's multiple range criterion ($P<0.05$).

Conclusion

In conclusion, the obtained results introduce a promising and innovative modality for malaria vector control using the photodynamic technique. These field studies are the results of three years of continuous

and persistent work, which have allowed the identification of different forms of mosquito-infected swamps as well as their access roads through the villages. The results of the field investigations have shown promising success in eliminating vectors over a short period of time by cutting the mosquito's lifecycle without formation of new generation or reinfestation. Cooperation with the local inhabitants, among whom awareness to this project was raised by instructing them on the official fieldwork procedure, will pave the road to future large-scale countrywide projects. This is our future prospect.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

Both authors have equally contributed to this work.

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Tables

Table 1: Criteria and environmental characteristics of the breeding sites.

S/N	Criteria of breeding sites selection		Sudan (Blue Nile State)	Uganda (Kampala city)	Ethiopia (Sodere village)
1	Nature of breeding sites	Temporal	√		
		Permanent		√	√
2	Environmental characteristics and close proximity		Within Agriculture fields near to village houses	Brick industry area near to Kebele houses	Brick industry area near to village houses
3	Physical variables	Water movement	Stagnant water	Stagnant water	Stagnant water
		Turbidity	Clear	Turbid	Turbid
		Sunlight exposure	√	√	√
		Solid wastes	No	No	No
4	Biological variables	Vegetation	√	No	No
		Potential predators	Tadpoles, aquatic snails and water insects	Tadpoles, *Schistosoma* snail vector and water insects	Tadpoles and water insects
5	Weather conditions	Temperature	30°C	22°C	20°C
		Humidity	53	70	50
		Rainfall	No	No	No
6	Season	Dry season	√	√	√
		Rainy season			
7	Breeding site area		2 - 5 m^2	5-10 m^2	5-12 m^2

Figures

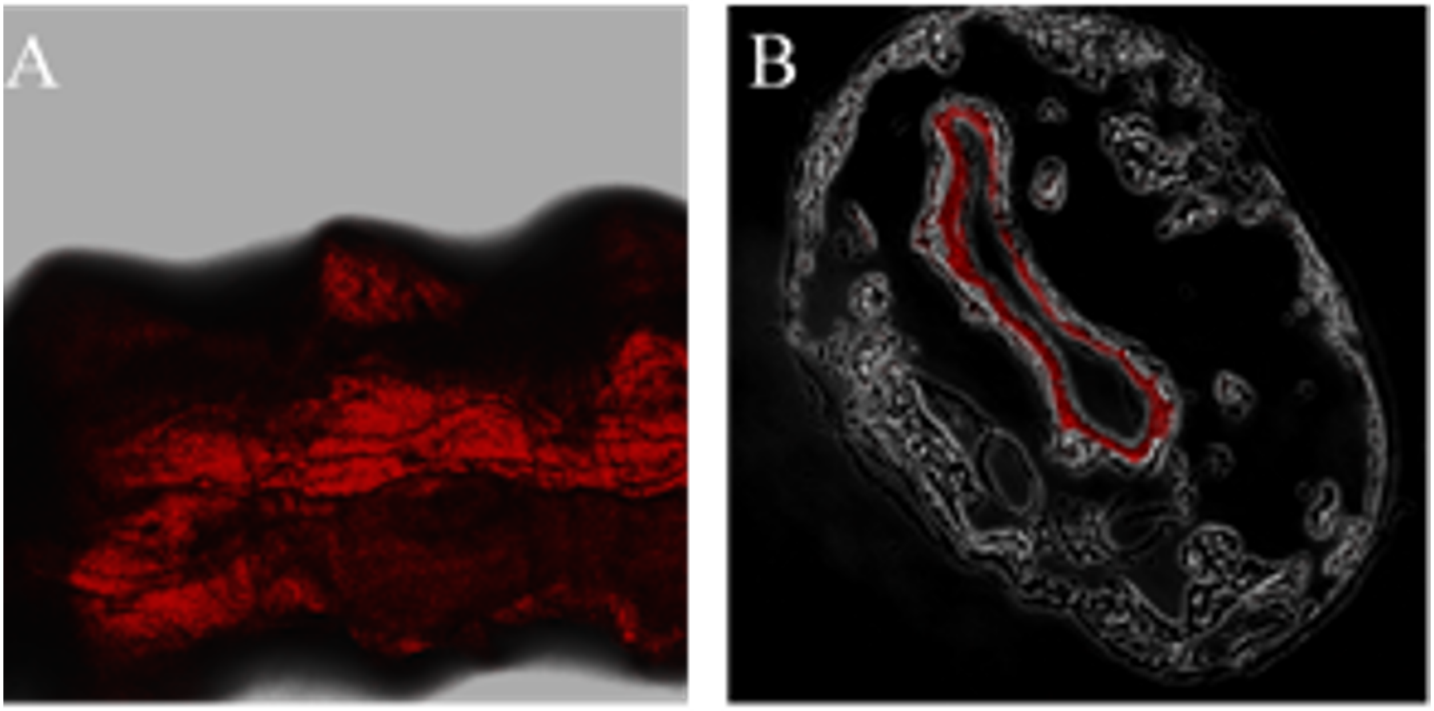
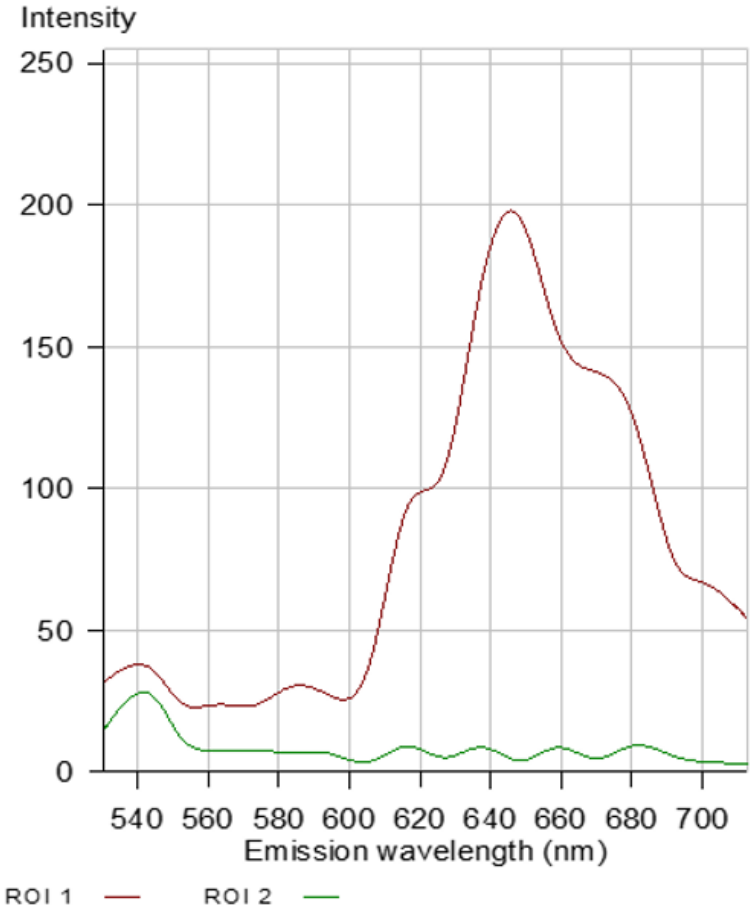


Figure 1

CLSM image of Anopheles larva of the whole mid-gut (A) and its transverse section (B) of Anopheles larva (200x).



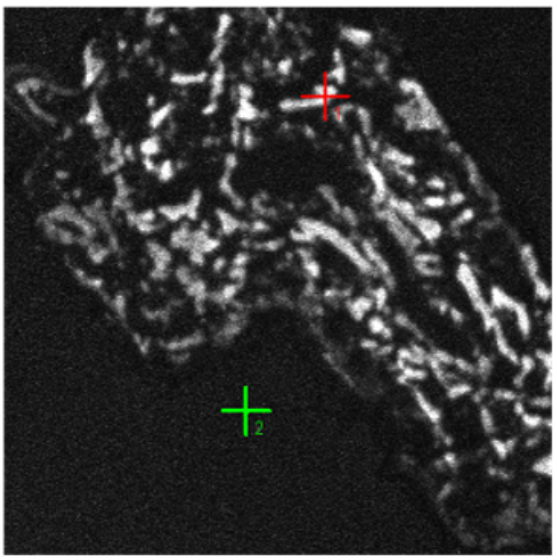


Figure 2

The relative intensity of chlorophyll in Anopheles larva tissue showed noticeable accumulation in different sites other than alimentary canal if it is compared with the background of CLSM image (200x).

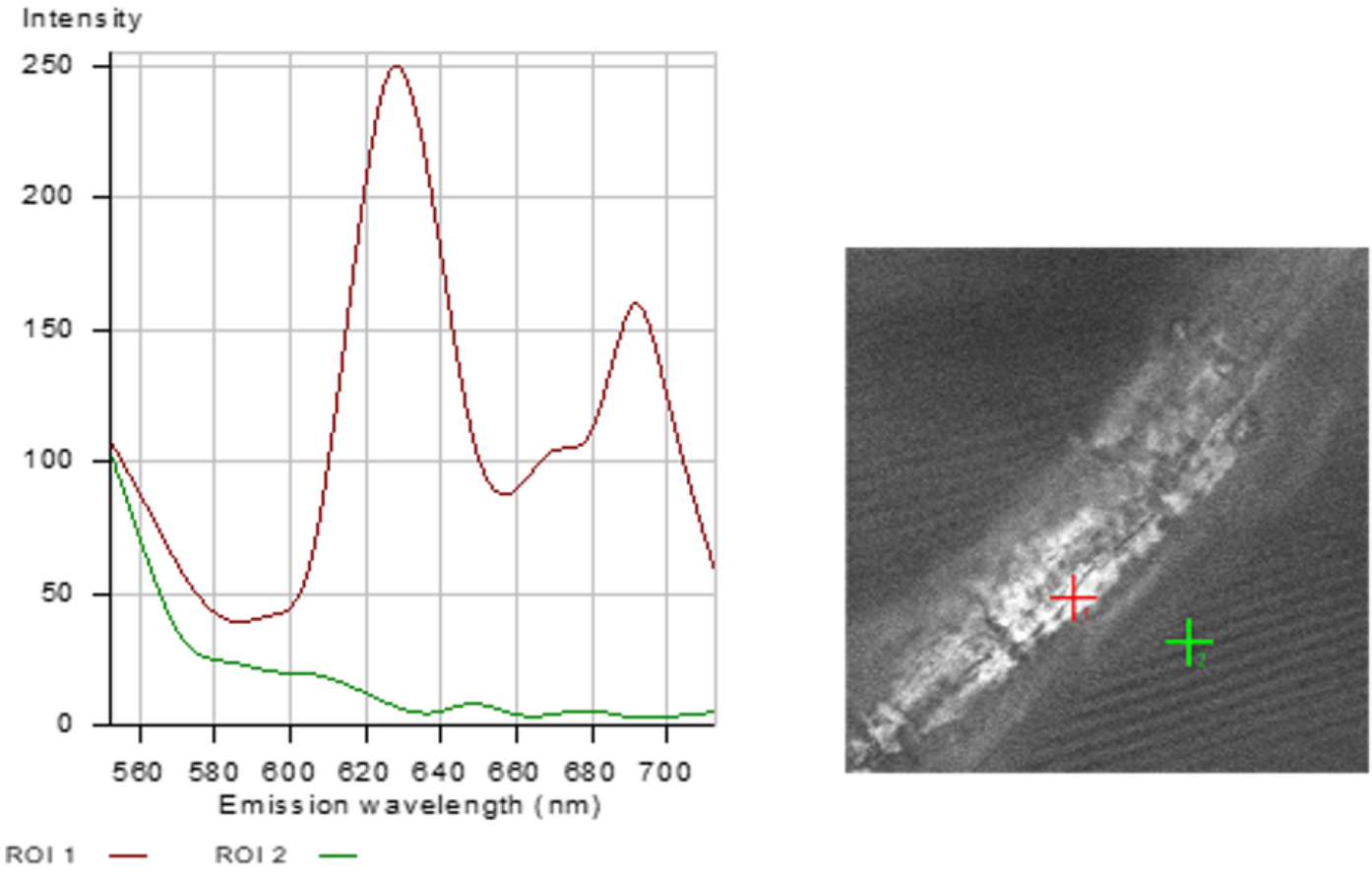


Figure 3

Ph-a fluorescence peak at 628 nm in the mid-gut of Anopheles larva (200x).

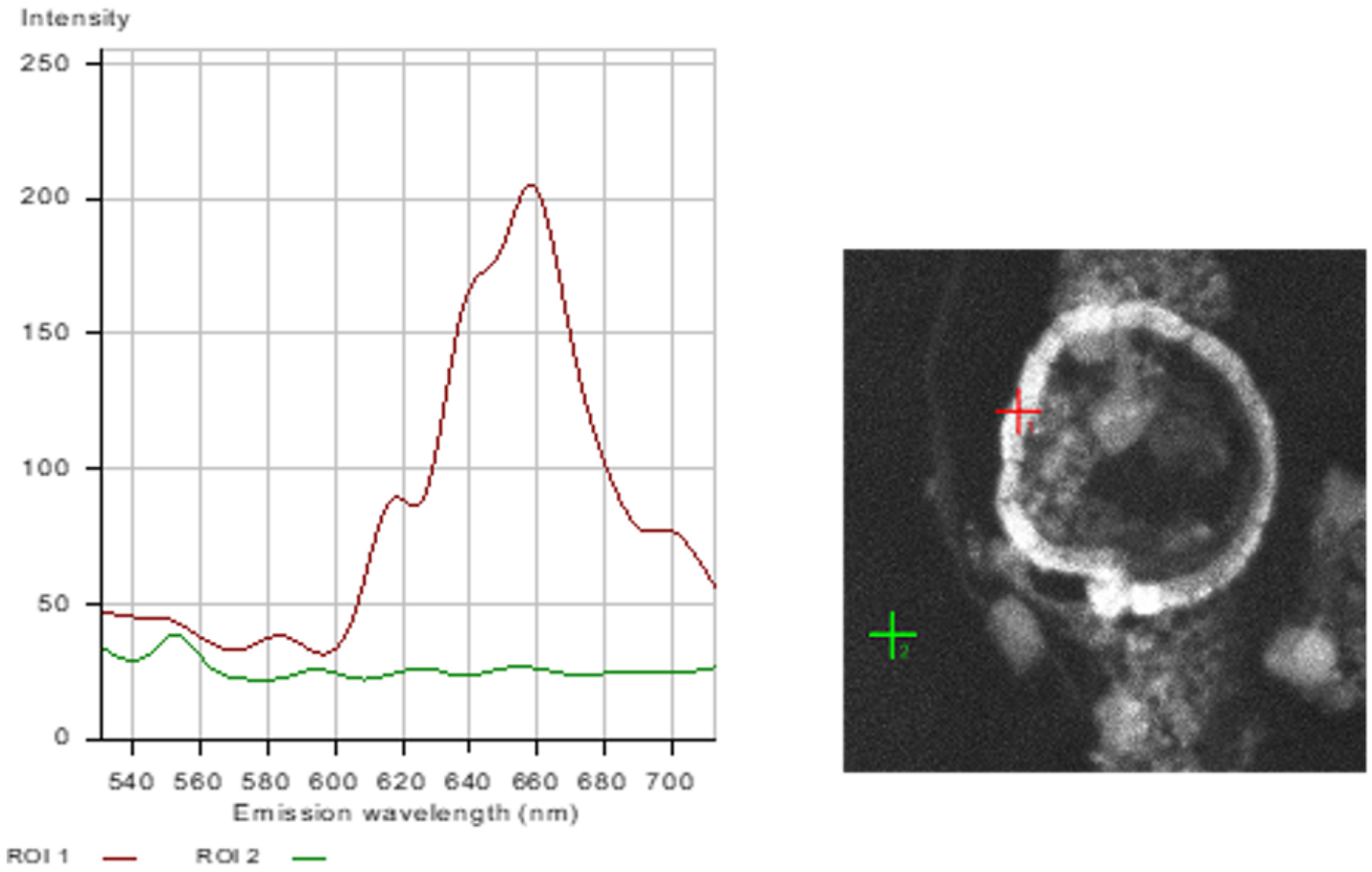


Figure 4

Ph-a fluorescence peak at 659 nm acquired from Anopheles larva transverse section (200x).

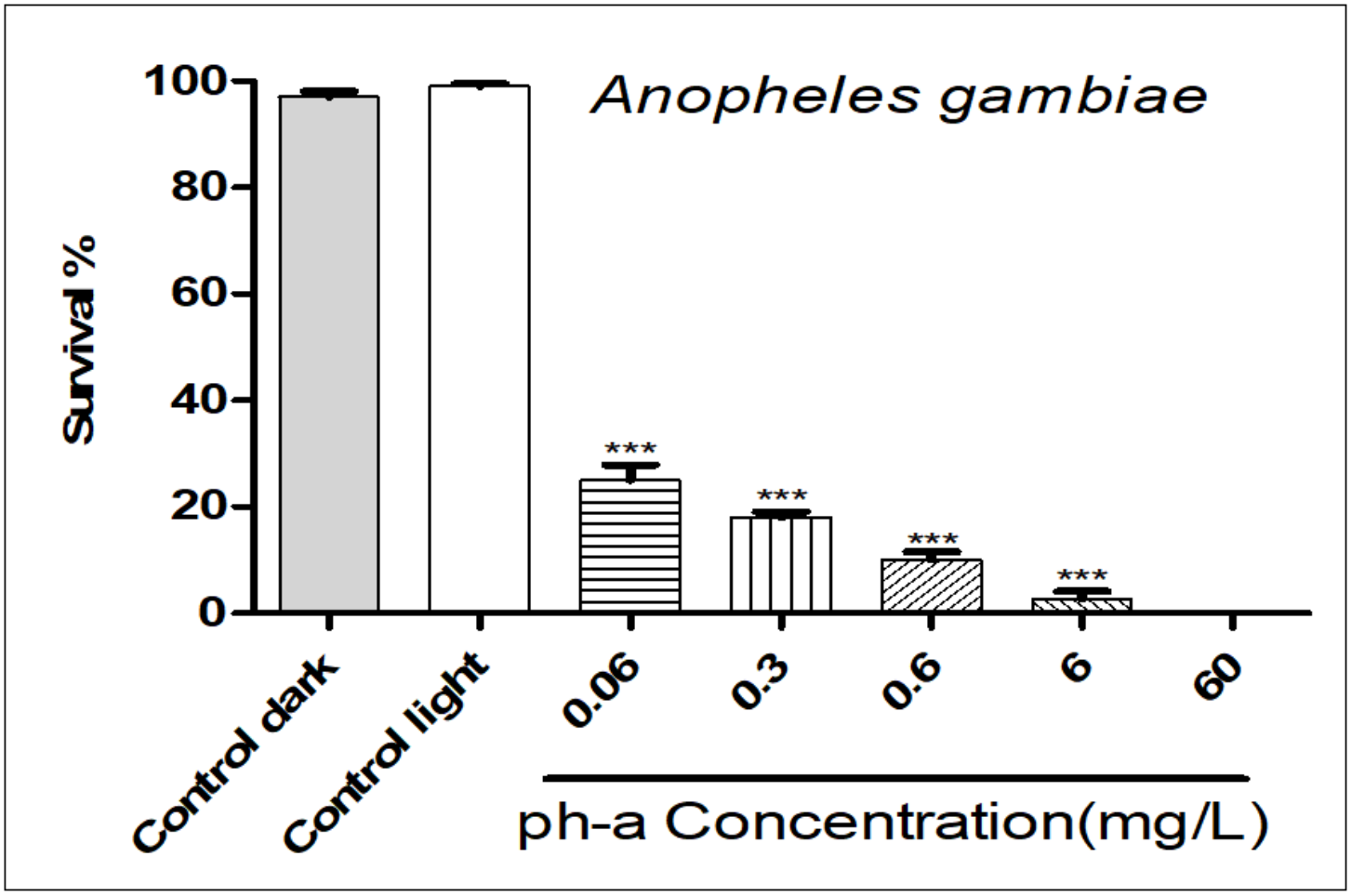


Figure 5

Effect of different chlorophyll derivatives concentrations (0.06 – 60 mg/L) on the percentage of survival of Anopheles gambiae: Field experiment in Sudan.

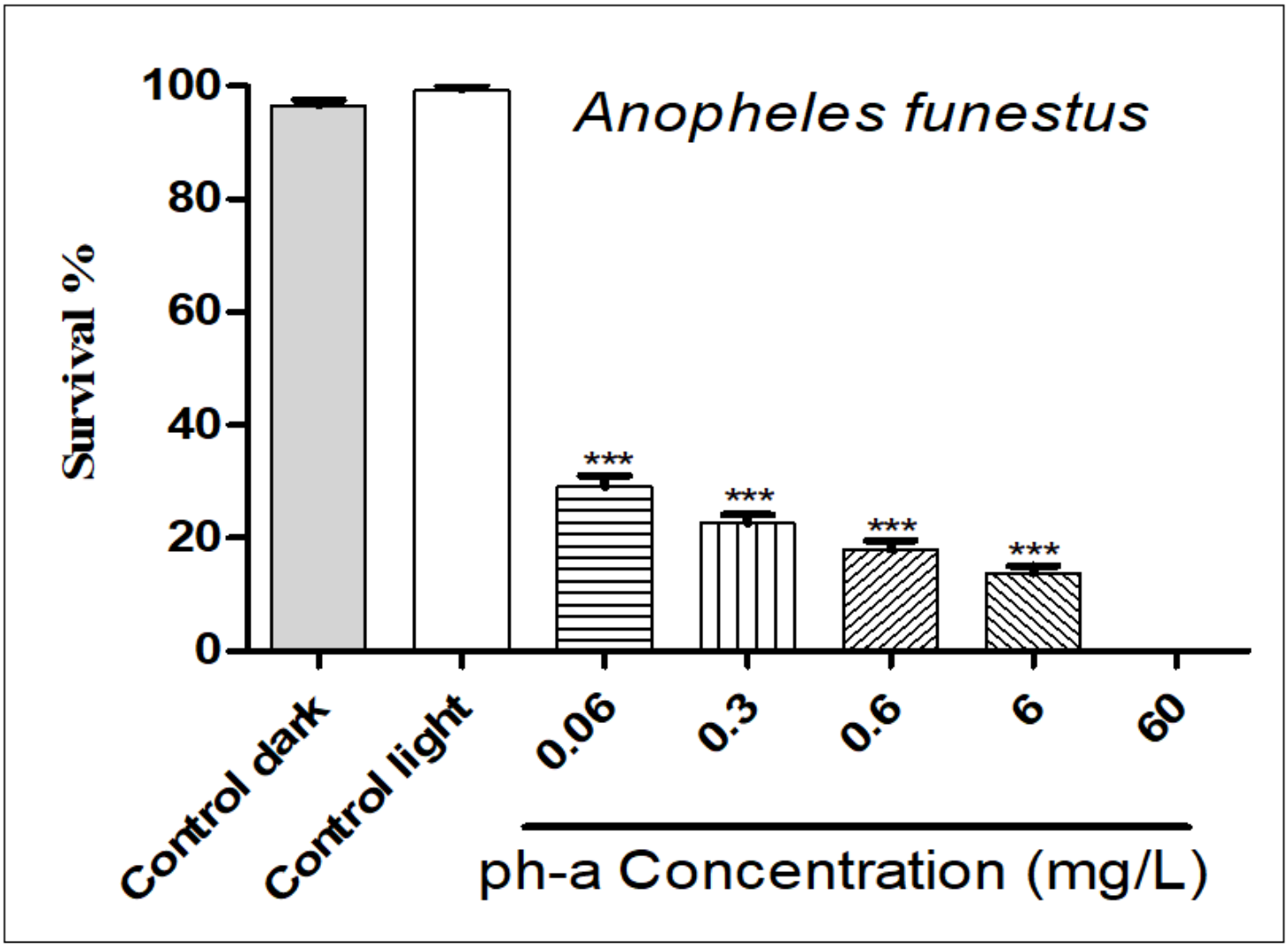


Figure 6

Effect of different chlorophyll derivatives concentrations (0.06 – 60 mg/L) on the percentage of survival of Anopheles funestus: Field experiment in Uganda.

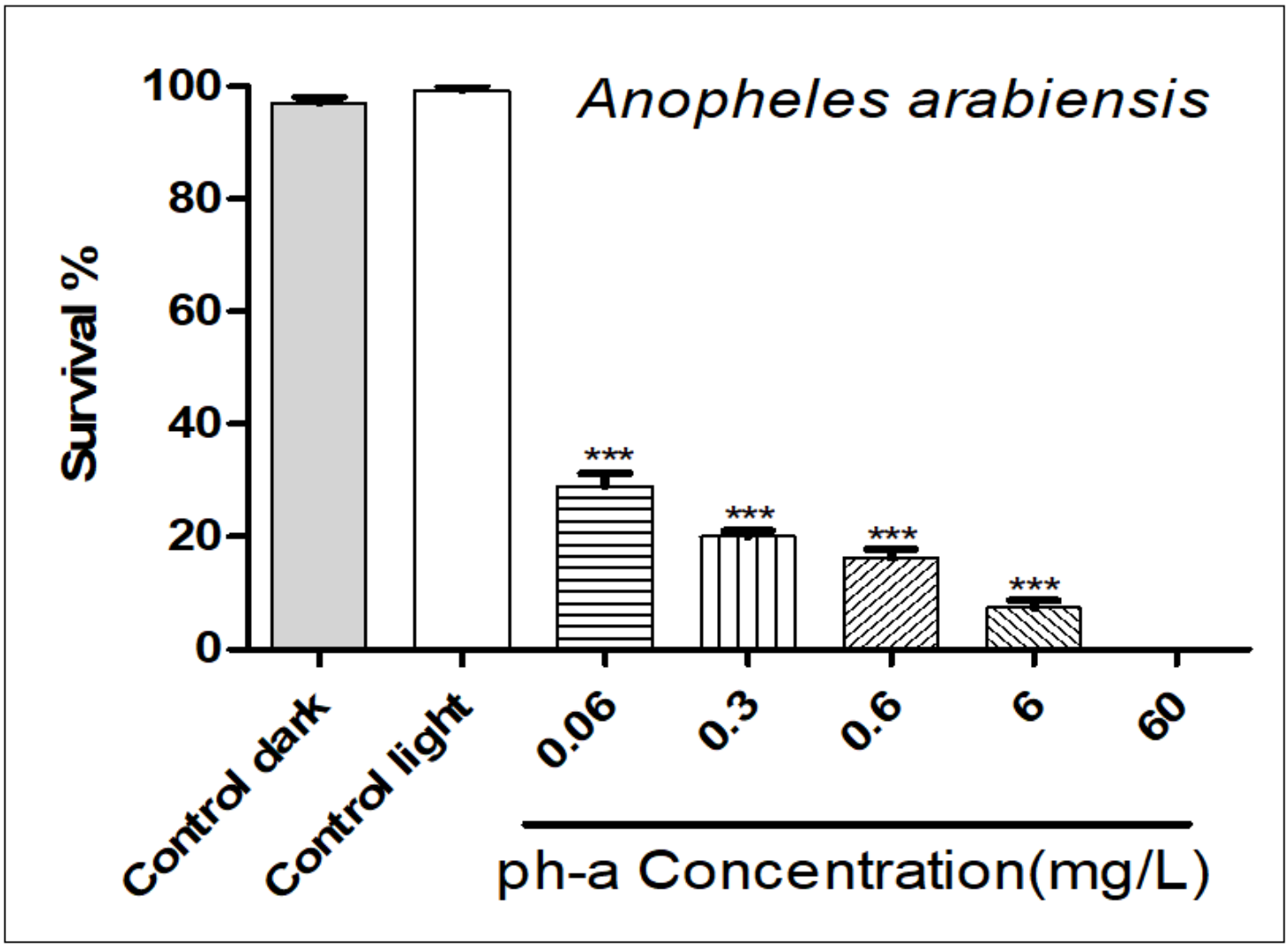


Figure 7

Effect of different chlorophyll derivatives concentrations (0.06 – 60 mg/L) on the percentage of survival of Anopheles arabiensis: Field experiment in Ethiopia.