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Genotoxicity of Chlorophyllin Compared with Other Pesticides Used to Control *Culex pipiens* Larvae (Diptera: Culicidae)

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ABSTRACT

Mosquitoes are prime examples of vectors for various diseases such as malaria, the West Nile virus, elephantiasis, dengue fever, and yellow fever. The repeated use of chemical pesticides has created numerous obstacles and environmental risks including mosquito resistance to insecticides. This work aimed at assessing the larvicidal effect of chlorophyllin (a water-soluble substance obtained after removing the phytol tail from chlorophyll) and coumarin as photosensitizers, compared to the microbial insecticide *Bacillus thuringiensis israelensis* (*Bti*) and permethrin, a chemical insecticide, against the third larval instar of *Culex pipiens* larvae monitoring its mode of action and its genotoxic effect. Photosensitizers exposed to sunlight generate reactive oxygen species (ROS), with singlet oxygen ($^1O_2$) capable of killing parasitic organisms primarily in aquatic systems. Our experiments demonstrated a high potential activity of Na-Cu chlorophyllin against mosquito larvae after at least 8 hours of incubation in the dark. The LC_{50} values were $0.22x10^{-3}$, $0.96x10^{-2}$, $13.7x10^{-2}$, and $4.59x10^{-1}$ mg/ l after 24 hours of exposure to chlorophyllin, *Bti*, permethrin, and coumarin, respectively. The molecular changes after treatment with chlorophyllin were tracked using RAPD-PCR with six arbitrary DNA primers. Results confirmed no significant changes or genetic damage after treatment with chlorophyll derivatives.

INTRODUCTION

Epidemic diseases such as dengue fever, malaria, and yellow fever are fundamental burdens that threaten hundreds of thousands of people, mainly in underdeveloped and developing countries, often hindering economic development in these regions **(Wohllebe *et al.*, 2009)**. Approximately 300 waterborne parasitic stages and 70 protozoan illnesses are diagnosed to have an impact on human beings, associated with aquatic vectors or intermediate hosts **(Cox, 2002)**. Mosquito larvae and numerous human parasites spend all or part of their life cycles in aquatic ecosystems. Climate

changes led to the invasion of the subtropical and tropical new water areas by such parasites, raising attention to the importance of it's control **(Goklany, 2004)**.

Mosquitoes are perfect examples of disease vectors. Of the identified 14,000 infectious microorganisms, six hundred are shared among animals and man kind. The Culex mosquito group can carry infectious disease agents, which include viruses and parasites. Mosquito-borne illnesses include malaria, the West Nile virus, elephantiasis, dengue fever, yellow fever and others **(Campbell *et al*., 2015; Kraemer *et al*., 2015)**. Special manipulation strategies have been developed in the past to eliminate these diseases and their vectors through therapeutic remedy and the usage of insecticides **(Rivero *et al.*, 2010)**. Certain pigmented substances can induce photodynamic activity. They are not toxic in the dark but are activated by light to produce a reactive triplet state. This state then reacts with oxygen to produce singlet oxygen ($^1O_2$), a cytotoxic molecule responsible for cellular apoptosis **(Peter *et al*., 2014)**.

The most important utility of those light-activated substances is the treatment of some vectors of diseases residing in contaminated water. In contrast to other insecticides, these cheap herbal substances such as chlorophyll or its derivatives (chlorophyllin and pheophorbide), can be easily prepared from plants **(Amor & Jori, 2000; Awad & Ragowsky, 2008; Ali *et al*., 2012; Erzinger *et al*., 2013)**. The hydrophobic properties of chlorophyll are treated to make it water-soluble by removing the phytol group responsible for hydrophobicity through alkaline treatment. Acidification of chlorophyllin produces a color change from green to olive yellow, indicating the formation of pheophorbide. Moreover, it is characterized by the absence of the central atom, magnesium, present in chlorophyll **(Tominaga & Caterina, 2004)**.

Chlorophyllin is a brand-new technique to control water parasites under exposure to sun **(Blaise *et al*., 2005)**. Its mode of toxicity is primarily based on the photodynamic interest of this colored natural molecule, after light activation, kills aquatic vectors of diseases **(He & Häder, 2002)**. Its degradation, without the formation of any toxic residues, makes this molecule economically and environmentally safe **(Wohllebe *et al*., 2011)**. The aim of this work was to add a new weapon to the arsenal of the non chemical trend of pest control strategies through assessing the larvicidal efficacy of chlorophyllin against *Culex pipiens* larvae and searching it's genotypic effect.

MATERIALS AND METHODS

Synthesis of Na-Cu chlorophyllin complexes

100mL of 65% ethanol was added to 30g of the leaf sample before being pulverized in a mortar and pestle. The homogenized sample was heated at 65°C in a water bath. 20mL of 5% NaOH was added to begin the saponification process for 20 minutes with stirring, which aids in clearing the cell of any remaining debris. The

samples were then centrifuged for 10 minutes at 4500rpm. After gathering the supernatant, 15mL of a 20% CuSO4 solution was added. After incubation for 30 minutes in the water bath, the Cu-chlorophyllin was extracted. Later, 2% NaOH solution was added to achieve pH 9.6 to produce soluble Na-Cu-chlorophyllin complexes. It was then centrifuged for 15 minutes at 4500rpm. The pellets were collected, allowed to dry, and stored in vials for further research **(Jubert *et al*., 2009)**.

Coumarin: 6,7-methoxy coumarin was obtained from Sigma-Aldrich.

***Bacillus thuringiensis israelensis*:** A Lab culture of *Bti* was obtained from the Microbiological Resources Center (Cairo MIRCEN) (Faculty of Agriculture, Ain Shams University) and was kept as a slant in our lab, and different concentrations were prepared for bioassay.

Permethrin was obtained as a stock solution from the Agricultural Research Institute, Al Dokki, Egypt Different serial concentrations in distilled water were prepared for bioassay.

Toxicity of chlorophyllin, coumarin, *Bti* and permethrin against *Cx. pipiens* larvae

Different concentrations of chlorophyllin solution, ($1x10^{-4}$, $1x10^{-3}$, $1x10^{-2}$, $1x10^{-1}$, $5x10^{-1}$ mg\ l) were used against twenty *Cx. pipiens* larvae (third instar) to test the solution's toxicity as a photo larvicide. For coumarin, concentrations of $5x10^{-2}$, $1x10^{-1}$, $5x10^{-1}$, $1x10^{0}$, $125x10^{-2}$ mg\ l were prepared for bioassay. *Bti* was applied at $1x10^{-4}$,$1x10^{-3}$,$1x10^{-2}$,$1x10^{-1}$, $10x10^{-1}$ mg\ l, while for permethrin $5x10^{-2}$,$1x10^{-2}$,$5x10^{-1}$,$1x10^{-1}$, $10x10^{-1}$ mg\ l were used as serial dilutions against mosquito larvae.

For bioassays, glass beakers with 100ml distilled water received 20 third instar larvae of *Cx. pipiens* and one ml of the tested toxins (chlorophyllin, coumarin, *Bti* and permethrin). Each concentration was repeated three times at least. Chlorophyllin was incubated in the dark for about 18 hours, then exposed to sunlight. All experiments were left at room temperature, moreover control experiments received distilled water only. Mortality readings were recorded after 24h and corrected to establish a regression line to calculate the lethal concentrations.

Calculating toxicity index and relative potency

Toxicity index formula **(Sun, 1950):**

$$\text{Toxicity index} = \frac{\text{LC50 of the most toxic insecticide}}{\text{LC50 of (less toxic) tested insecticides used}} \times 100$$

$$\text{Relative potency} = \frac{\text{LC50 for least used insecticide competence (Lowest toxic)}}{\text{LC50 for other insecticides tested}}$$

RAPD PCR conditions (Yang & Quiros, 1993)

The bulked DNA of healthy and treated *Cx.pipiens* larvae were extracted using DNeasy Mini Kit (QIAGEN). The DNA amplification cycles were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94ºC for 4min followed by 45 cycles of 1min at 94ºC, 1min at 57ºC, and 2min at 37ºC. The reaction was finally stored at 72ºC for 10min. Agarose gel electrophoresis was used to characterize the product. Ethidium bromide was used as a dye to visualize DNA bands. Primers used during amplification with its sequences are tabulated in Table (1).

Table 1. List of the primer sequences used for RAPD-PCR

Primer name	Sequence
OP-A5	CCTTGACGCA
OP-A9	CCTTGACGCA
OP-B3	CAT CCC CCT G
OP-C9	CTC ACC GTC C
OP-C15	GAC GGA TCA G
OP-D1	ACC GCG AAG G

The similarity index reflects the extent of band sharing and calculated as 2Nab\Na+Nb, where Nab is number of bands common to individuals a and b, while Na and Nb are total number of bands in a and b **(Nei & Li, 1979)**.

RESULTS

1. Susceptibility of 3rd instar *Cx. pipiens* larvae to chlorophyllin, coumarin, *Bti*, and permethrin.

The potency of chlorophyllin, coumarin, *Bti*, and permethrin were measured against *Cx pipiens* larvae. The mortality readings, LC 50 and 90s, toxicity indices and relative potencies after 24 and 48h post treatment were tabulated in Tables (2, 3)

Table 2. Toxicity of four toxicants against 3rd instar *Cx. pipiens* larvae after 24h (bioassay)

Toxic compound	Concentration (mg\l)	Mortality (%)	LC50(mg\l)	LC90(mg\l)	Slope	Toxicity index based on LC_{50} values	LC90\ LC50	Relative potency levels based on LC_{50} values
Chlorophyllin	$1x10^{-5}$	26.7	$0.22x10^{-3}$ ($0.9x10^{-3}$-$4.7x10^{-3}$)	$6x10^{-2}$ ($350x10^{-2}$-$1000x10^{-2}$)	0.46	100	272.7	181.81
	$1x10^{-4}$	46.7						
	$1x10^{-3}$	58.3						
	$1x10^{-2}$	76.7						
	$5x10^{-2}$	88.3						
Coumarin	$5x10^{-3}$	13.3	$4.59x10^{-1}$ ($2.75x10^{-1}$-$4.76x10^{-1}$)	$5.6x10^{-1}$ ($3200x10^{-3}$-$12600x10^{-3}$)	0.919	0.55	14	1.00
	$1x10^{-2}$	21.7						
	$5x10^{-2}$	53.3						
	$1x10^{-1}$	71.7						

	$12.5x10^{-1}$	81.7						
Bti	$1x10^{-5}$	13.3	$0.96x10^{-2}$ ($4.9x10^{-3}$-$189x10^{-3}$)	$760x10^{-2}$ ($150x10^{-2}$-$1100x10^{-2}$)	0.55	2.29	159.25	4.17
	$1x10^{-4}$	26.7						
	$1x10^{-3}$	56.7						
	$1x10^{-2}$	68.3						
	$1x10^{-1}$	86.7						
Permethrin	$1x10^{-3}$	15	$13.7x10^{-2}$ ($8.8x10^{-2}$-$21.3x10^{-2}$)	$3.4x10^{-2}$ ($310x10^{-2}$-$4920x10^{-2}$)	13.38	2.20	50	4.00
	$5x10^{-3}$	27.5						
	$1x10^{-2}$	51.7						
	$5x10^{-2}$	66.7						
	$1x10^{-1}$	80						

Table 3. Toxicity of four toxicants against the 3rd instar larvae of *Cx. pipiens* after 48h (bioassay)

Toxic compound	Concentration (mg\L)	Mortality (%)	LC50(mg\l)	LC90(mg\l)	Slope	Toxicity index based on LC_{50} values	LC90\LC50	Relative potency level based on LC_{50} values
Chlorophyllin	$1x10^{-5}$	40	$1.8x10^{-4}$ ($1x10^{-4}$-$800x10^{-4}$)	$5x10^{-2}$ ($12.3x10^{-2}$-$307x10^{-2}$)	0.44	100	500	777.78
	$1x10^{-4}$	58.3						
	$1x10^{-3}$	76. 7						
	$1x10^{-2}$	86.7						
	$5x10^{-2}$	90						
Coumarin	$5x10^{-3}$	26.7	$14x10^{-2}$ ($10x10^{-2}$-$120x10^{-2}$)	$1x10^{-1}$ ($5.1x10^{-1}$-$104.7x10^{-1}$)	1.50	0.13	0.71	1.00
	$1x10^{-2}$	41.7						
	$5x10^{-2}$	76.7						
	$1x10^{-1}$	**90**						
	$12.5x10^{-1}$	**95**						
Bti	$1x10^{-5}$	26.7	$3x10^{-4}$ ($8x10^{-4}$-$3.5x10^{-4}$)	$15x10^{-2}$ ($0.508x10^{-2}$-$1.0470x10^{-2}$)	0.55	60	277.78	466.76
	$1x10^{-4}$	41.7						
	$1x10^{-3}$	66.7						
	$1x10^{-2}$	81.7						
	$1x10^{-1}$	95						
Permethrin	$1x10^{-3}$	15	$1x10^{-2}$ ($0.88x10^{-2}$-$0.213x10^{-2}$)	$1.7x10^{-1}$($0.31x10^{-1}$-$4.93x10^{-1}$)	0.90	0.90	17	14.00
	$5x10^{-3}$	27.5						
	$1x10^{-2}$	51.7						
	$5x10^{-2}$	66.7						
	$1x10^{-1}$	80						

Results in Tables (2, 3) prove that the mortality rates were directly dependent on concentrations in all selected pesticides. The maximum toxic effect was shown in treated larvae with chlorophyllin at concentration $5x10^{-2}$ mg\ l, followed by *Bti* ($1x10^{-1}$ mg\L), permethrin ($1x10^{-1}$ mg\l) and coumarin ($12.5x10^{-1}$ mg\l).

Probit analysis (Table 2) revealed that LC_{50} after 24 hours' post- exposure was ($0.22x10^{-3}$ mg\ l) for chlorophyllin, ($0.96x10^{-2}$ mg\ l for *Bti*, $13.7x10^{-2}$mg\ l for permethrin and

$4.59x10^{-1}$ mg\ l for coumarin. The results indicate that chlorophyllin was found to be the most effective extract, while coumarin was the least effective one. Data in Table (2) also show that the toxicity indices of coumarin, *Bti*, and permethrin were 0.55, 2.29, and 2.20%, respectively, compared to chlorophyllin at the LC50 value against *Cx. Pipiens.* According to relative potency levels, the toxicity of chlorophyllin, *Bti*, and permethrin were 181.81, 4.17, 4.00 folds as toxicity of coumarin against *Cx. pipiens*.

Probit analysis (Table 3) reveals that LC_{50} values after 48 hours' post- exposure was $1.8x10^{-4}$ mg\ l for chlorophyllin, $3x10^{-4}$ mg\ l for *Bti,* $1x10^{-2}$ mg\ l for permethrin and $14x10^{-2}$ mg\ l for coumarin. LC_{90} values of the tested pesticides were calculated as $5x10^{-2}$ mg\ l for chlorophyllin, for *Bti* it was $15x10^{-2}$ mg\ l, $1.7x10^{-1}$ mg\ l for permethrin and $1x10^{-1}$ mg\ l for coumarin. Chlorophyllin was the most effective photoactive larvicide, while coumarin was the least effective one. Data in Table (3) also show that the toxicity index of coumarin, *Bti*, and permethrin were 0.13, 60, 0.90% compared with the toxicity of chlorophyllin at LC50 value. According to the relative potency levels, the toxicity of chlorophyllin, *Bti*, and permethrin were 777.78, 466.76, 14.00 folds as the toxicity of coumarin against *Cx. pipiens*.

Figures of bands:

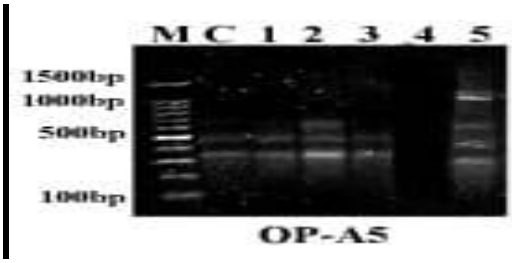

Fig. 1. RAPD-PCR produced for *C. pipiens* larvae using primer OP-A5

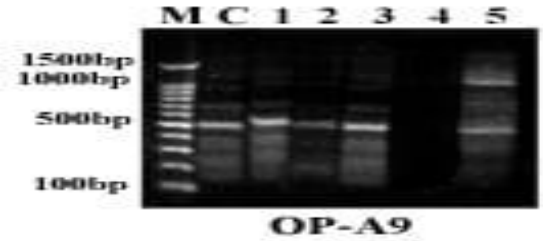

Fig. 2. RAPD-PCR produced for *C. pipiens* larvae using primer OP-A9

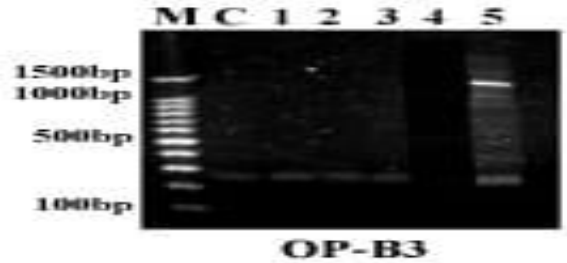

Fig. 3. RAPD-PCR produced for *C. pipiens* larvae using primer OP-B3

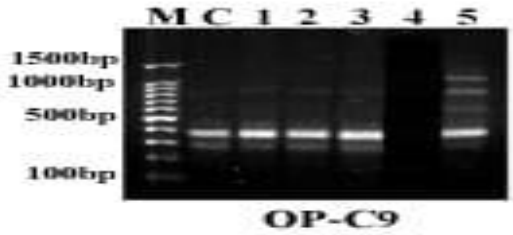

Fig. 4. RAPD-PCR produced for *C. pipiens* larvae using primer OP-C9

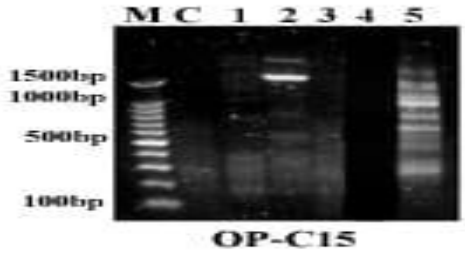

Fig. 5. RAPD-PCR produced for *C. pipiens* larvae using primer OP –C15

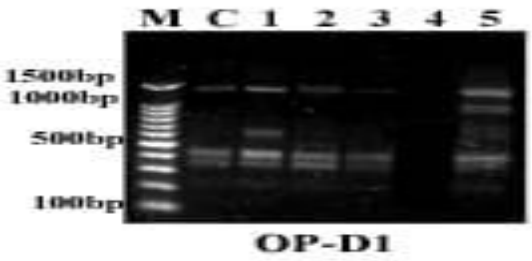

Fig. 6. RAPD-PCR produced for *C. pipiens* larvae using primer OP-D1

Table 4. Total number and size of RAPD-PCR fragments generated by arbitrary primers in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-A5

	Band 1	Band 2	Band 3	Band4	Band5	Band6	Band 7	Band 8	Total	Similarity index
Control							340	270	2	-
Chlorophyllin							340	270	2	1
Bti						640	340	270	3	0.80
Coumarin							340	270	2	1
Permethrin	2260	1875	1340	1230	1060	640	340	270	8	0.50
Frequency	0.17	0.17	0.17	0.17	0.17	0.5	1	1		
Polymorphism	Unique	unique	Unique	Unique	Unique	Polym-orphic	Mono-morphi-c	Mono-morphi-c		

Table 5. Total number and size of RAPD-PCR fragments generated by arbitrary primers in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-A9

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	Band9	Band10	Total	Similarity index
Control			1385	1080	870		735	400	380	260	7	-
Chlorophyllin			1385	1080		760	735	400	380	260	7	0.86
Bti			1385	1080		760	735	400	380	260	7	0.86
Coumarin			1385			760	735	400	380	260	6	0.71
Permethrin	**2385**	1760	1385		870	760		400	380	260	7	0.71
Frequency	0.17	0.17	1	0.5	0.33	0.83	0.67	1	1	1		
Polymorphism	Unique	Unique	monomorphi-c	Polymorphi-c	Polymorphi-c	Polymorphi-c	Polymorphi-c	Monomorphic	Monomorphic	Monomorphic		

Table 6. Total number and size of RAPD-PCR fragments generated by arbitrary in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-B3

	Band1	Band2	Band3	Band4	Band5	Band6	Total	Similarity index
Control					485	300	2	-
Chlorophyllin					485	300	2	0.67
Bti						300	1	0.67
Coumarin						300	1	0.50
Permethrin	2280	1485	1260	945	485	300	6	0.50
Frequency	0.17	0.17	0.17	017	0.67	1		
Polymorphism	Unique	Unique	Unique	Unique	Polymorphic	Monomorphic		

Table 7. Total number and size of RAPD-PCR fragments generated by arbitrary primers in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-C9

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	total	Similarity index
Control	1355			825		630	400	300	5	-
Chlorophyllin	1355			825		630	400	300	5	1
Bti				825		630	400	300	4	0.80
Coumarin	1355			825	785	630	400	300	6	0.80
Permethrin	1355	1270	1080	825	785		400	300	6	0.60
Frequency	0.83	0.17	0.17	1	0.33	0.83	1	1		
Polymorphism	Polymorphi-c	Unique	Unique	Monomorph-ic	Polymorphi-c	Polymorphi-c	Monomorph-ic	Monomorph-ic		

Table 8. Total number and size of RAPD-PCR fragments generated by arbitrary primers in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-C15

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	Band9	Band10	Total	Similarity index
Control				930	760	650		365		245	5	-
Chlorophyllin	2730	1765	1075	930	760		470	365	315		8	0.46
Bti	2730	1765	1075	930	760		470	365	315		8	0.46
Coumarin		1765		930	760		470		315	245	6	0.54
Permethrin		1765	1075	930	760	650	470	365	315	245	9	0.71
Frequency	0.33	0.83	0.67	1	1	0.5	0.83	0.83	0.83	0.67		
Polymorphism	Polymorphi-c	Polymorphi-c	Polymorphi-c	Monomor-phic	Monomor-phic	Polymorphi-c	Polymorphi-c	Polymorphi-c	Polymorphi-c	Polymorphi-c		

Table 9. Total number and size of RAPD-PCR fragments generated by arbitrary primers in *Cx. pipiens* larvae treated with LC_{50} values using primers OP-D1

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	Total	Similarity index
Control		1355			635	460	375		4	
Chlorophyllin		1355			635	460			3	0.86
Bti		1355	1275		635	460			4	0.75
Coumarin		1355	1275	845	635	460		235	6	0.60
Permethrin		1355	1275	845	635	460	375	235	7	0.80
Frequency	0.17	1	0.67	0.5	1	1	0.5	0.5		
Polymorphism	Unique	Monomorphic	Polymorphic	Polymorphic	Monomorphic	Monomorphic	Polymorphic	Polymorphic		

RAPD- PCR fingerprint profiles were generated using different primers to track the behavior of genomic DNA of *Cx. pipiens* larvae after treatment with chlorophyllin, coumarin, *Bti*, and permethrin. The obtained results showed that the fingerprints generated in the treated larvae by each primer revealed polymorphic, monomorphic, and unique profiles for treated and untreated larvae. The used primers gave good amplification with distinct fragmentsو as shown in Tables (4- 9) and Figs. (1- 6).

PCR results using the primer OP-A9 showed DNA bands for untreated larvae at 1385, 1080, 870, 735, 400, 380 and 260bp. Treatment with chlorophyllin proved a slight variation than normal, with a similarity index= 0.86. This treatment was characterized by the disappearance of 870bp band and appearance of new band at 760bp. Treatment with coumarin showed the disappearance of DNA bands at 1080 and 870bp and gave a similarity index of 0.71. The effect of treatment with chlorophyllin and *Bti* on DNA, using OP-A9, was similar and showed the same patterns, with the same similarity index of 0.86. Nevertheless, treatment with permethrin gave different unique DNA patterns at 2385 and 1760bp with a similarity index =0.71.

DNA profile of healthy larvae, using OP-B3 as a primer during RAPD-PCR, appeared two bands at 485 and 300bp. The same bands appeared after treatment with chlorophyllin, which show a similarity index identical to untreated larvae. Treatment with coumarin and *Bti* showed the same deviation as normal with the disappearance of the 485bp band and similarity indices of 0.67 and 0.67.

Using the primer OP-C9, during RAPD- PCR technique proved the similarity in DNA patteren between healthy and chlorophyllin treated larvae, with a similarity index =1. Treatment with *Bti* led to the disappearance of 1355bp band, while treatment with coumarin resulted in the appearance of new band at 785bp, beside the original fragments (1355, 825, 630, 400 and 300bp). Treatment with permethrin after DNA configuration showed a weak similarity index (0.6).

When using OP-C15 as a primer, DNA configuration after treatment with chlorophyllin showed a greater difference than the normal with the appearance of three new bands of high molecular weights (2730, 1765 and 1075bp) and disappearance of 650bp band. Its similarity index changed to 0.46. The behavior of DNA treatment with *Bti* was similar to that appeared after chlorophyllin treatment. Treatment with coumarin was characterized by the appearance of a new band at 245bp and the disappearance of 365bp band. Treatment with permethrin resulted in a similarity index of 0.71.

After using the primer OP-D1, as shown in Table (9), great similarity between untreated and chlorophyllin treated DNA with the disappearance of the band 375bp was detected. Treatment with *Bti* appeared a new polymorphic band at 1275bp and disappearance of the band at 375bp and showed similarity index as 0.75. While, the treatment with coumarin resulted in three new bands compared to the untreated specimen at 1275, 845, and 235bp with the disappearance of 375bp.

DISCUSSION

Control of disease vectors by means of photo activity of some green plant derivatives (photosensitizers) is taken into consideration as one of the most secure, effective, and ecologically friendly tactics for controlling vector-of epidemic and endemic illnesses **(Abdel-Kader & Eltayeb, 2014)**. Photosensitizers use light to produce reactive oxygen species (ROS). This singlet oxygen ($^1O_2$) has the potential to kill parasitic organisms in particular in aquatic systems **(Wohllebe *et al*., 2011)**. Numerous synthetic and obviously derived photosensitizers together with xanthenes, thiophenes, phenothiazines, acridines, furocoumarins, tetraethynylsilanes and porphyrins were found to efficaciously kill quite a range of insects consisting of mosquitoes, as cited by **Lucatoni *et al*. (2011)**.

The search for more cost effective and environmentally friendly photosensitizers to be used for pests and vectors control led to the exploration of photodynamically active chlorophyll derivatives. Chlorophyll derivatives like chlorophyllin (water-soluble obtained after removal of the phytol tail from chlorophyll) and pheophorbide (obtained from chlorophyllin by acidification) have proven to efficiently kill the larvae of several pests and snails as well as certain parasites of aquatic organisms by exerting a strong photodynamic effect **(Wohllebe *et al*., 2009; Abdel-Kader *et al.*, 2012; Erzinger *et al.*, 2013; Mahmoud *et al*., 2013)**. Exposure to chlorophyllin or pheophorbide in dark for a few hours and next light illumination brought about oxidative pressure that bring about necrosis and apoptosis inside the frame of some arthropods and snails **(Wohllebe *et al*., 2011)**, chlorophyll derivatives had been implemented and proved its interest toward a few mosquitoes spp. **(Wohllebe *et al*., 2009; Erzinger *et al*., 2013)**. Chlorophyllin was reported to efficiently kill the eggs and larvae of different freshwater snails, the intermediate hosts of some zoonotic diseases **(Mahmoud *et al*., 2013)**. Chlorophyllin was

found to be approximately 100 times more effective than other photosensitizers, such as methylene blue and hematoporphyrin **(Wohllebe *et al.*, 2009)**.

Based on a field trial in some African countries, **Abdel-Kader and El-Tayeb, (2012)** recommended that chlorophyll derivatives can be successfully used to control the vectors of human diseases such as malaria, filarial and dengue fever.

Due to the promising results obtained at laboratory level as well as in a few field trials, the application of chlorophyll derivatives as a measure against pests and disease vectors is getting wide attention and may become a common practice in the near future. This concept is further supported by low cost since chlorophylls can easily and economically be extracted (from numerous plant sources) and converted to its effective derivatives like chlorophyllin and pheophorbide **(Wohllebe *et al.*, 2009)**. It is necessary to ensure that any substances added to the environment are safe, i.e. it should not accumulate in the place of application or negatively influence its ecological structure. Chlorophyll is known to be readily degraded and does not lead to the accumulation of any toxic intermediate or product in the environment **(Heaton & Marangoni, 1996)**. In a laboratory test, chlorophyllin and pheophorbide did not show any adverse effect on adult fish and the larvae of some invertebrates, viz. *Chironomus* and the european snail species **(Wohllebe *et al.*, 2009)**. It was also claimed in a field trial that chlorophyll derivatives were target selective and killed only targeted (mosquito's) larvae, while non-target organisms, such as the larvae of dragon fly and mosquito predators live in the treated swamps, were not affected **(Abdel-Kader & El-Tayeb, 2014)**. Countless studies were needed to guarantee safety of these compounds to nontarget organisms in the environment. **Wohllebe *et al.* (2011)** declared that certain non-target organisms like daphnia and fish larvae were adversely affected by chlorophyllin and pheophorbide. Generally, it is believed that photosensitizers do not cause any toxicity to humans or other animals with non-translucent bodies since toxicity of these molecules is mainly dependent on photodynamic reactions **(Lucatoni *et al.*, 2011)**.

The cytotoxicity of pheophorbide as a chlorophyll derivative in mouse model was reported in the study of **Wohllebe *et al.* (2011)**. Intact chlorophyll cannot easily penetrate the cell and is not toxic, but its derivatives like pheophorbide and chlorophyllin due to lack of phytol tail can penetrate the cell and exert toxicity **(Wohllebe *et al.*, 2011)**.

There are few studies concerning the effects of chlorophyll derivatives on the cellular level, physiological parameters and DNA damage when used against mosquito larvae. It is important to measure such parameters, which may explain larval death as a result of treatment and killed by photosensitizing compounds. This study focused on the impact of photosensitizers, microbial pesticides on DNA compared with the effect of using permethrin as a chemical pesticide. It can be concluded that chlorophyl derivatives caused *Cx. pipiens* larval death via the effect on biochemical, physical, and physiological

parameters of mosquito larvae with no significant effect on DNA. Normal DNA molecules, after the larval treatment, reflected no chance for the undesired mutation of the sublethal concentration of chlorophyll derivatives. Oxidative stress phenomenon appeared as a result of disproportion between active oxygen species generation and cellular neutralization systems including enzymatic and non-enzymatic, mediated pathways. The imperative antioxidant enzyme systems regulate reactive oxygen species (ROS) quantity at a physiologically tolerable state to keep redox homeostasis in its normal cellular level. The most promising application of such derivatives is the ease of its application in parasite-contaminated water; these inexpensive natural substances, chlorophyll, and its derivatives (chlorophyllin and pheophorbide) can easily be extracted from plants. Application of traditional chemical pesticides cannot be a good choice now owing to their toxicity, risk to humans and domestic animals. Toxicity of photosensitizers is based on photodynamic reactions; no toxicity of chlorophyll derivatives was monitored after being digested by humans or other animals due to their non-translucent bodies **(Lucatoni *et al.*, 2011)**. Photosensitive chlorophyllin is degraded extremely fast without the formation of toxic byproducts; therefore, it is environmentally sound and economically safe, but more experiments, particularly on light attenuation in breeding places of mosquitoes, are needed. The results of genotoxicity revealed that permethrin induced obvious mutagenic effect at high and low doses, while chlorophyllin induced a slight genotoxicity at high dose only.

CONCLUSION

Chlorophyll derivatives may not be as safe to the environment as they are believed to be and can possibly pose a risk to non-target organisms. Although several groups across the world are working on the exploration of chlorophyll derivatives as alternative to chemical pesticides for controlling pests and disease vectors, rare attention has been given to the evaluation of their toxicity to non-target organisms. To the best of our knowledge, no previous study has dealt with the ecotoxicological assessment of chlorophyll derivatives. Therefore, the search of environmental biologists, particularly ecotoxicologists, is needed to fill this gap and facilitate the field application of chlorophyll derivatives. It can be concluded that permethrin can induce prominent genotoxicity while chlorophyllin shows a low side effect on DNA.

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical statement

All experiments in this research were approved by the Ethics Committee of the Faculty of Science, Ain Shams University, Cairo, Egypt (Approval code: ASU-SCI/ENTO/2023/6/4).

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