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The activity of *Bacillus thuringiensis* Vectobac G on the biochemical compositions of *Culex pipiens* and *Culiseta longiareolata* (Diptera: Culicidae) mosquito larvae

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ABSTRACT

Culex pipiens and Culiseta longiareolata, are domestic mosquitoes, and ones of the most dominant and abundant mosquito species in the urban areas of Algeria. Thus, the present study was conducted to evaluate the larvicidal activity of *Bacillus thuringiensis* (Bt.) Vectobac G and to estimate the disturbance of main body metabolites in the tissues of fourth instar larvae of Cx. pipines and Cs. longiareolata. The toxicological essays were done using different concentration of Bti vectobac G against a new exuviated fourth instar larve of Cx. pipiens, and Cs. longiareolata for 24h of exposure time. The results showed a toxic effect with a concentration-response relationship of this product. The biochemical analyses were carried out with the estimated lethal concentrations of both used species (Cx. pipiens: LC50=0.81 µg /l and LC90=3.57 µg /l; Cs. longiareolata: LC50=1.80 µg/l and LC90=5.43 µg/l) on the fourth instar larvae. As a result, Bti. Vectobac G caused a significant decrease in the whole body weight in the treated larvae as compared to the control series. Moreover, biochemical analyses revealed an increase in carbohydrates levels, and a decrease in protein and lipids in the whole larvae body treated with the lethal concentrations as compared to the control series for the both species Cx. pipiens and Cs. longiareolata.

Key words: *Culex pipiens, Biological control, Bacillus thuringiensis, Metabolites.*

Introduction

Mosquitoes are a major concern for entomologists around the world due to their role in transmitting several diseases to humans, such as malaria, yellow fever, dengue, West Nile, Zika, Chikungunya fever, Japanese encephalitis, and lymphatic filariasis (Govindarajan and Benelli, 2016; Nuttall, 2020; Mahamane *et al.,* 2020). Whose disease-transmission power occurred mainly by blood-sucking bite, which has been well-documented (Hay *et al.,* 2002). Further, the success of insecticide-based control programs in reducing the prevalence of vector-borne diseases with costs economically relevant for devel-

oping regions is irrefutable (Curtis and Davies, 2001). The large repeated uses of these expensive synthetic products arise some disadvantages such as the insect resistance development, disrupted natural biological control systems which led to resurgence of these pests, undesirable effects on environment, non-target organisms, and on human health (Richards *et al.,* 2020; Smith and Perfetti, 2020; Jalapathi *et al.,* 2020). However, the stringent pesticide registration procedures that are needed to minimize their negative ecological and health impacts have reduced the number of chemicals available for vector control (WHO, 2006) have encouraged the search to find alternatives; specific and non-polluting substances like Bio insecticides, such as *Bacillus thuringiensis*. Moreover, combined efforts of the World Health Organization (WHO), research laboratories, and several international programs were oriented for the study the effects of *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* (*Bs*) (Barloy *et al.,* 1996). Since different *Bt* varieties were proposed as specific to some insect orders including Lepidoptera, Diptera, and Coleoptera (Sanda *et al.,* 2018; Ghazwan *et al.,* 2017). Toxins from *Bacillus thuringiensis* bacteria have appeared as an efficient alternative to conventional insecticides for mosquito control (Boudjelida *et al.,* 2008; Bonizzoni *et al.,* 2013; Aissaoui and Boudjelida, 2014). Research works have confirmed that *Bacillus thuringiensis* var. *israelensis* (*Bti*) could be as good alternative according to the toxic effects on mosquito larvae (Aissaoui and Boudjelida, 2014; Gonzales *et al.,* 2019), while Their biological activities have not yet been well assessed. Therefore, the present study was carried out to evaluate the insecticidal effect of *Bt.* Vectobac-G on the against domestic mosquito *Cx. pipiens* and *Cs. longiareolata,* which are representing the most important (Aissaoui and Boudjelida, 2017) and abundant mosquito species in Algeria (Aroussi *et al*., 2021) and to evaluate the effect of the lethal concentrations (LC_{50} and LC_{90}) on biochemical parameters like the whole body weight and metabolite contents (carbohydrates, proteins and lipids) of the fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata* and subsequently to compare the effect of *Bti* Vectobac G on the both mosquito.

Materials and Methods

Mosquito strains

The larvae of *Culex pipiens* and *Culiseta longiareolata* were obtained from laboratory colonies. Larvae were reared in storage jars containing 500 ml of stored tap water and maintained at temperature between 25-27 °C and a photoperiod of 14:10 (L:D). They were daily fed with fresh food consisting of a mixture of Biscuit-dried yeast (75:25 by weight), and water was changed every tree days. During pupal stage, the pupae were transferred to other jars containing 500 ml of water with the help of a dipper and were kept in mosquito cage for adult emergence.

Insecticide and bioassay

Laboratory bioassays were conducted on the efficacy of *Bacillus thuringiensis* Vectobac G (active ingredient [AI]: 300 *Bt* international toxic units [ITU]/ mg product) at different concentrations, against newly exuviated fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata*. *Bt.* Vectobac G consisted of a lyophilized powder of spores and crystal mixture of lysed cultures of *Bt.* For testing, serial dilutions from the product were made from the stock to obtain the appropriate range of concentrations. For each vector species and the formulation was tested at 4 different concentrations (0.25, 0.75, 1.25, 2.5 µg/l) for both mosquito species. The test was carried out with three repetitions containing 25 larvae each. The treated larvae series were exposed to the water-dispersible granule (G) formulation *Bt*. Vectobac G for 24 hours as described by the World Health Organization (WHO, 2005). Than the water was changed and the food was added every three days until the emergence of the adults. Direct mortality is expressed as the % mortality recorded after 24 hours exposure of the treated larval stage. Cumulative mortality is the total mortality scored after treatment of the larval stages up to adult emergence.

Extraction of metabolites

Extraction of the main metabolites (lipid, carbohydrate, and protein) has been performed as described elsewhere (Shibko *et al.,* 1967). The bodies pool of 25 newly exuviated larvae of the fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata* were treated with the lethal concentrations of the Formulation (LC_{50}=0.81 µg /l, LC_{90}=3.57 µg/l; LC_{50}= 5.43 µg/l, LC_{90}=1.80 µg/l respectively) and homogenized in 1ml of trichloroacetic acid (TCA 20%). After first centrifugation (5000 rpm, 10 min), the obtained supernatant was used to evaluate the carbohydrate, and then pellet 1 was added to the mixture of ether and chlo-

roform (1V / 1V). Another supernatant resulting from the second centrifugation was used for lipid quantification (Goldsworthy *et al.,* 1972), while pellet 2 would be dissolved in soda (0.1 N) and used to evaluate the protein contents. Data were expressed in µg/mg of the whole body and expressed as means ± SD. Pairwise comparisons were analyzed based on three replicates per treatment using a Student's t-test, where $p < 0.05$ was considered significant. Analyses were conducted on survivors after treatment.

Biochemical procedure

Quantification of proteins was carried following the Bradford method (1976) with bovine serum albumin as a standard and Coomassie Brilliant Blue as reagent. The absorbance was measured at 595nm. Carbohydrates were determined following the Duchateau and Florkin method (1959) using glucose as standard and anthrone as reagent. Lipids were measured by the Goldsworthy method (1972) using sunflower oil as standard and vanillin as reagent.

Statistical Analysis

The mortality percentage observed for each concentration subjected to correction using the Abott formula (Abbott, 1925) when the mortality was observed in the control series and subjected to probit analysis (Finney, 1971). Data from larvicidal tests were subjected to analysis of variance after angular transformation of observed mortality percentages than the regression curves were made and from where LC_{50}, LC_{90}, at 95% confidence limits and the slope were estimated (Swaroop *et al.,* 1966).

Results

Insecticidal activity

Bioassays showed that *Bt.* Vectobac G has a high toxicity against species, *Cx. pipiens* and *Cs. longiareolata*. Results are summarized in figures 1 and 2 and represent the effect of the toxin against newly ecdysed larvae. Mortality occurred directly on treated larvae and also a cumulated action until adult emergence. The reported results were subjected to the analysis of variance with repeated measures, which reveals a highly significant ($p<0.000$) concentration-response relationship effect for the both species of mosquito. Under the lower concentration of 0.25µg/l the mortality mean was 24% for *Cx. pipiens*, while no deaths were recorded for *Cs. longiareolata* (Figure 1). Using the highest concentration of 2.5 µg/l of the *Bt.* Vectobac G the mortality increased for the both species of mosquito, when it was 89.33% for *Culex pipiens* and 60% for *Cs. longiareolata* (Figure 1).

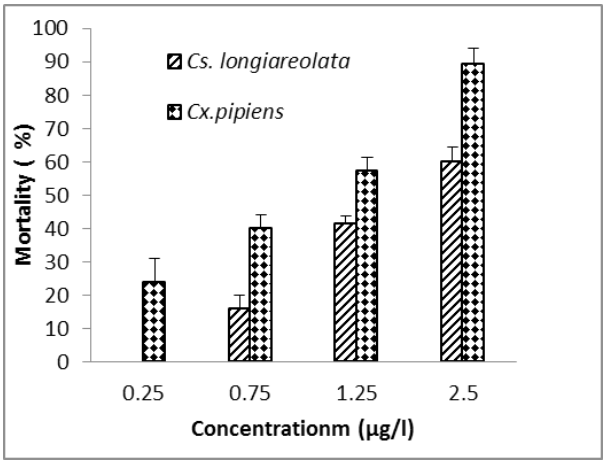

Fig. 1. Concentration-response relationship for treatment of *Bt*. Vectobac G applied to the newly exuviated fourth instar larvae of *Culex pipiens* and *Culiseta longiareolata.*

The target mosquito species tested were extremely sensitive to the *Bt.* Vectobac G formulation with the most sensitive species being *Cx. pipiens*. With probit analysis, the LC_{50} and LC_{90} were calculated and the confidence limits for all stages were estimated (Table 1).

The effect of Bt. Vectobac G on the Fresh Body Weight

As indicated in table 1, both of the tested lethal concentrations (LC_{50} and LC_{90}) of *Bt* Vectobac G have remarkably affected the whole bodyweight of the newly ecdysed fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata*. In the control series, the larval body weight was 5.33± 0.15mg and then it continuously decreased to reach the minimum weight on the 7th day with 3,86± 0,05 mg for *Cx. pipiens*. Furthermore, the whole body weight of larvae of *Cs. longiareolata* was significantly decreased after treatment with the two lethal concentrations (LC_{50} and LC_{90}) of the active material (Table 2). Also, a significant decrease in the bodyweight of the treated *Cs. longiareolata* larvae was noticed from the third day of the two tested concentrations, meanwhile, the highest body weight recorded on the 7th day was only 6.23 mg for LC_{50} (P= 5.43) and 5.03 mg for LC_{90} (P= 0.81) as compared

Table 1. Toxicity of *Bt*. Vectobac G against *Culex pipiens* and *Culiseta longiareolata larvae* (LC_{50}, LC_{90}, LCL, UCL µg/l)

Species	Linear regression	Slope	LCL>LC_{50}>UCL (µg/l) Confidence limit (95%)	LCL>LC_{90}> UCL (µg//l) Confidence limit (95%)
Cx. pipiens	y = 1.9973x + 1.2078	3.01	0.68<0.81<0.95	4.21<3.57<3.02
Cs. longiareolata	y = 2.6805x – 1.0571	2.34	2.42<1.8<1.42	6.84<5.43<4.30

Table 2. Effect of *Bt.* Vectobac G administered with two lethal concentrations (LC_{50} and LC_{90}) on the fresh body weight (mg) during the fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata* (means ± SD; n=3).

	Body weight (mg/Larva) of the 4th instar larvae					
	Culex pipiens			Culiseta longiareolata		
Treatment Day	Control	$LC_5$00.81µg/l	LC_{90}3.57µg/l	Control	LC_{50}1.80 µg/l	LC_{90}5.43 µg/l
1	3.16±0.28	2.43±0.15	1.90±0.36*	5.30±0.52	4.06± 0.51	4.20±0.52
3	4.06±0.25	2.43±0.40*	2.86±0.11*	6.40±0.36	5.36±0.41*	4.53±0.46*
5	4.96±0.28	3.43±0.20**	3.16±0.20**	7.63±0.28	5.43±0.28**	5.16±0.60**
7	5.33±0.15	4.40±0.26*	3.86±0.05**	7.60±0.17	6.23±0.32 *	5.03±0.25**

*p<0.05: Significant difference, and ** p<0.001: A highly significant difference from the control of the same exposure time.

with controls. No significant difference (P> 0.05) in the weight of both mosquito species of fourth instar larvae between the two different tested lethal concentrations from the 5th day was observed.

Effects on Biochemical Composition

In the second series of the experiment, *Bt* Vectobac G was tested with the lethal concentrations (LC_{50} and LC_{90}) on the variations of the metabolite amounts (carbohydrates, lipids, and proteins) of the fourth instar larvae of *Cx. pipiens* and *Cs.longiareolata* during different times of the developmental stages (1,3, 5 and 7days).

***Bt.* Vectobac G Effect on the total protein content**

As shown in figure 2, treatment with *Bt.* Vectobac G significantly decreased the total protein contents in the whole body extracted from 4th larvae stage of *Cx. pipiens* and *Cs. longiareolata*; for both concentrations, since they were found significantly higher in *Cx. pipiens* than those in *Cs. longiareolata.* The total protein contents following treatment with *Bti* at LC_{50} and LC_{90} were respectively, 40.16 and 38.88 mg, but on the first day of the control group, the protein levels were 45.50 mg in *Cx. pipiens*. Likewise, the decrease in the level of proteins in *Cs. longiareolata* at

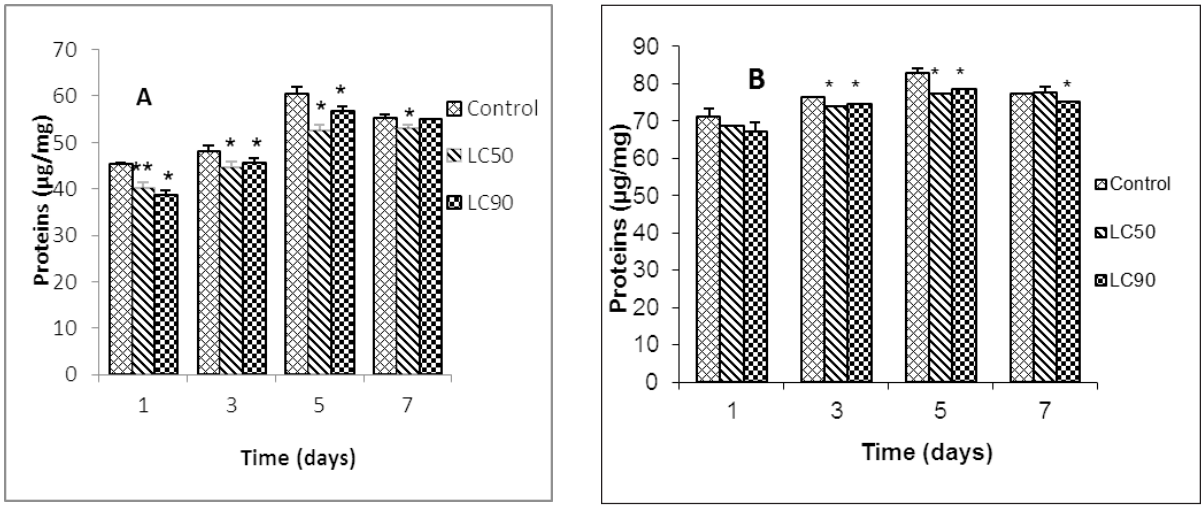

Fig. 2. Effect (LC_{50} and LC_{90}) of Bt. Vectobac G on the protein contents in the larval body (µg /mg body weight) during the fourth larval stage development of Cx. pipiens (A) and Cs. longiareolata (B).

the 7th day was significant only for the LC_{90} (75.33 mg) when compared with the control series. In addition, the highest level of protein contents was noticed in the whole-body extract of *Cs. longiareolata* from the fourth larval stage compared to those in *Cx pipiens.*

Bt. Vectobac G Effect on the total lipid content

The levels of lipids were evaluated in the whole body extracts from the fourth *Cx. pipiens* and *Cs. longiareolata* larvae subjected to treatment with *Bt.* Vectobac G at lethal concentrations (LC_{50} and LC_{90}). The comparison of mean values shows that the lipid contents in *Cs. longiareolata* decreased significantly ($p<0.001$) with the highest concentration (LC_{90}) (87±1.73 µg/mg) during the 5th day when compared with the control group (101.67±2.89 µg/mg). During the 3rd and 5th days, the lipid levels decreased significantly only for LC_{50} (indicated by a shift-pick) in *Cx. pipiens* and *Cs. longiareolata* with a shift pick (Figure 3).

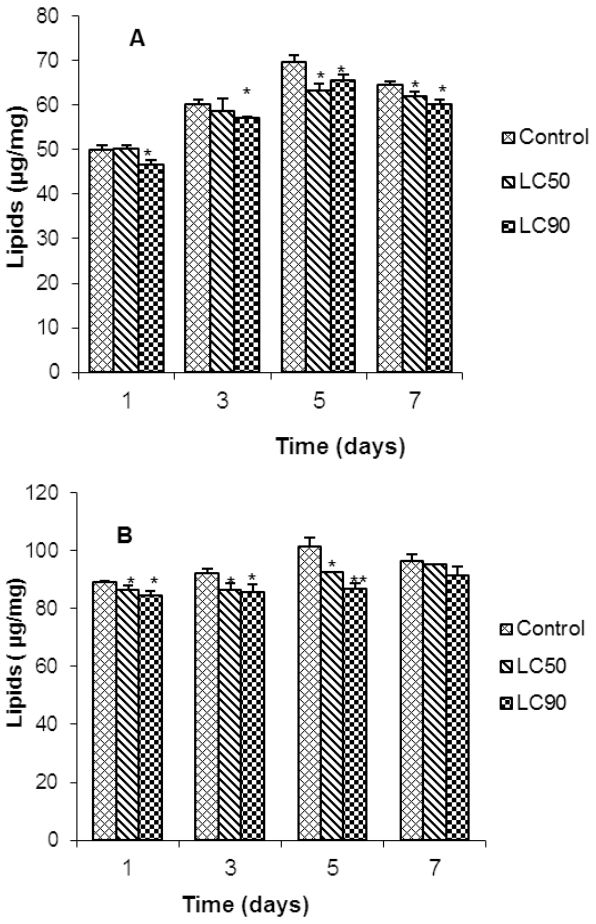

Fig. 3. Effect of (LC_{50} and LC_{90}) *Bt.* Vectobac G on lipid contents in the larval body (µg /mg body weight) of *Cx. pipiens* **(A)** and *Cs. longiareolata* ***(B***) during the fourth larval stage of development.

Bt. Vectobac G Effect on the total carbohydrate contents

As shown in figures 4 (A and B), the difference in the carbohydrate contents in *Bt.* Vectobac G (LC_{50} and LC_{90}) treated larvae of *Cx.pipiens* and *Cs. longiareolata* were significantly lower (P=0.004) on the 3th day as compared to control ones. Whereas, *Bt.* Vectobac G treated larvae were found to undergo a significant ($P< 0.05$) decreased level during the periods of bioassays 5 and 7 days when compared with the control series. Additionally, the rate of the decreased amount of carbohydrates in *Cx. pipiens*

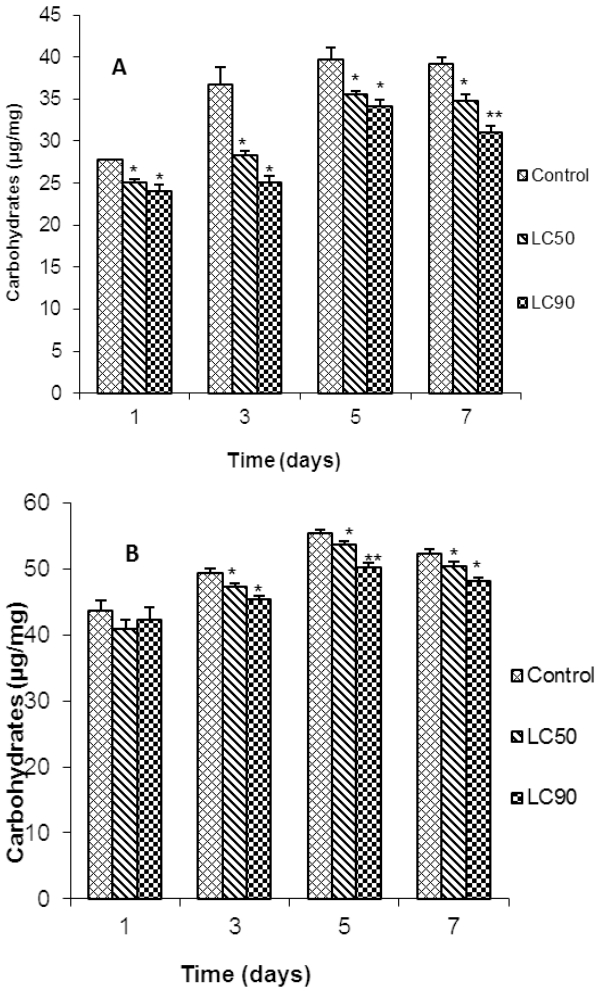

Fig. 4. Effect of (LC_{50} and LC_{90}) *Bt.* Vectobac G on carbohydrate contents in the larval body (µg /mg body weight) of *Cx.pipiens* **(A)** and *Cs. Longiareolata* (B) during the fourth larval stage development.

was higher than that noticed in *Cs. longiareolata* for both concentrations, and was highly significant with LC_{90}. The results of the treated series revealed that the levels of carbohydrates, lipids, and proteins shift significantly from the 1st day up to the 7th day.

Dicussion

Larvicidal activity

Mosquito vectors are generally controlled by conventional neurotoxic insecticides (Cuervo-Parra *et al.,* 2016) and growing public concern over environmental pollution, use of chemical insecticides is no longer encouraged; instead of that use of effective and environment friendly alternatives are promoted. From this point of view, the isolation and evaluation of the new isolates as potential mosquito control agents are becoming vitally essential (Ghosh *et al.,* 2019). Many research works carried out to evaluate *Bacillus thuringiensis* impact against different natural pests have been done (Aissaoui and Boudjelida, 2014; Gonzales *et al.,* 2019; Dunstand-Guzman *et al.,* 2020). This study emphasizes the biological control of *Cx. pipiens* and *Cs. longiareolata* (Diptera: Culicidae) mosquito and the effects using a new bacterial bio-larvicides. Subsequently, the toxicity assays conducted under laboratory conditions on *Cx. pipiens* and *Cs. longiareolata* larvae indicated that the new variety of *Bti.* Vectobac G exhibited a larvicidal activity when applied to newly ecdysed larvae for 24 h. The same effects were found when the *Bacillus sphaericus* was used against *Anopheles stephensi* (Kumar *et al.,* 2013).

So far in the treatment, the target mosquito species tested were extremely sensitive to the *Bt*. Vectobac G formulation, with the most sensitive species: *Cx.pipiens* than *Cs. longiareolata.* Efficacy and safety characteristics of this bacterial agent have made it a suitable candidate for large-scale production and field-testing. The larvicidal activity of the locally isolated *Bacillus thuringiensis* strain against the larvae of *Culex quinquefasciatus* was optimum at around 35C° temperature. The larvae were found to be more susceptible against *Bt* in its natural habitat, as providing supplementary food has helped the larvae to combat against the larvicidal activity of the *Bacillus thuringiensis* (Ghosh *et al.,* 2019).

Fresh Body Weight

This study was carried out to provide an introductory understanding of the significant role of *Bti.*Vectobac G against *Cx.Culex pipiens* and *Cs. longiareolata* mosquitoes. In this study the effect of *Bt.*Vectobac G (LC_{50} and LC_{90}) on the fourth instar larvae showed that treatment of selective extractions disrupts the biochemical composition by reducing proteins, lipids and the fresh body weight and increasing carbohydrates of *Cs. pipiens* and *Cs. longiareolata* larvae at the fourth stage as compared with controls. In similar works (Bouaziz *et al.*, 2011; Djeghader *et al.,* 2014) demonstrated that the Insect Growth Disruptive (IGD or IGR), novaluron also significantly reduced the body weight of the larvae of the fourth larval stage of *Cx. pipiens*. Similarly, *Ruta graveolens* essential oil causes a reduction in weight of body in *Cx. pipiens* (Dris and Bouabida, 2020). Moreover, the haemolymph of insects undergoes metabolic modification during its developmental stages (Cohen, 2010). Since the exposure of an organism to xenobiotic products can modify the synthesis of certain metabolites and disturb their functionality. The ability of a mosquito to survive, and hence to transmit diseases depends largely on its caloric reserves (Van Handel, 1984), as well as the blood meal volume, and the number of mature eggs produced are affected by the body volume of the females mosquito (Briegel, 1990).

Biochemical Composition

In insects, the main body metabolites, Carbohydrates, protein and lipids, have an essential role in different biological and physiological activities, such as body size, growth rate, fecundity and fertility (Fagan *et al.,* 2002). In physiological studies, the determination of total protein and many chemical macromolecules such as lipids and carbohydrates is important. The bioassays of the mean metabolites reveal that there are changes in the levels of carbohydrates, lipids and proteins in the whole body of the treated larvae of *Cs. longiareolata* and *Cx. pipiens* during different periods of larval development in comparison with control. The same result of a pevious work (Bouguerra *et al.,* 2018) showed a significant decrease in protein, carbohydrate and lipid levels in fourth instar larvae of *Cx. pipiens* treated with the *Thymus vulgaris* essential. The biochemical analyses of the constituents realized in the whole body of *Cx. pipiens* and *Cs. longiareolata* larvae after treatment with the lethal concentrations (LC_{50} CL_{90}) of *Bt.*Vectobac G the comparison of protein contents in both species proved that the *Bt.*Vectobac G is ef-

fective on both of them. The results of the metabolite assays of the treated fourth larvae indicated a decrease in the levels of proteins in the whole body of the treated. compared to the control series. The comparison of protein contents in both species proved that the *Bt.*Vectobac G is effective on both of them. The reduction of protein is thereby explained by the protein degradation in metabolic ways or by the impaired incorporation of amino acids into polypeptide chains or inhibition of protein synthesis. In this opinion, Tanani *et al.,* (2021) reported that the reduction of protein content in larvae and pupae of *Galleria mellonella* after larval treatment with the tested arthropod venoms, can be interpreted in the light of some conceivable suggestions: "(1) the proteins might be due to their binding with the tested arthropod venoms and therefore might reflect the depressed activity of the detoxifying enzymes. (2) that the protein plays a major role in synthesis of the microsomal detoxifying enzymes against toxicants (foreign compounds) entering into the insect body (Hassan, 2002). (3). protein metabolism plays a key role in rebuilding adult structures during the transformation of larvae/pupae into adults in insects, (Resmitha *et al.,* 2014)". Furthermore, the decrease in these proteins has been reported in some previous studies, including those investigating the effect of Novaluron (IGD) on *Cs. longiarealata* (Bouaziz *et al.,* 2011), and the effect of The tested *Leiurus quinquestriatus* venom, *Vespa orientalis* venom or Apitoxin of *Apis mellifera* which might either act on the hormonal regulation (disruptive) of the protein synthesis, degradation and inhibition or act on the neurosecretory cells which control endocrine organs (Djeghader *et al.,* 2014 ; Tanani *et al.,* 2021).

Accordingly, the results showed a marked decrease in the total lipid contents in *Bti.*Vectobac G treated *Cs. longiareolata* and *Cx. pipiens* during the tested larval stage, likewise the effect of Novaluron on *Culiseta longiareolata* larvae (Bouaziz *et al.,* 2011). The decrease of the lipid level Concorde with those obtained after treatment with some insect growth regulators (Soltani-Mazouni *et al.,*1999; and Bouaziz *et al.,* 2011). On the other hand, all the venoms of *Leiurus quinquestriatus*, *Vespa orientalis* and *Apis mellifera* exhibited prevalent enhancing effects on larvae to gain more lipids than control larvae of both 5th and 7th instars of *galleria mellonella*. In fact, the increase of lipid content during treatment may be due to the high accumulation of carbohydrates, which could be metabolized into lipids to be stocked in the form of reserves (Tanani *et al.,* 2021).

In general carbohydrate and lipid levels increase during rest periods, like metamorphosis, and decrease during the growth periods like the stages of insect gonads maturation, yet carbohydrates play a key role in the physiology of those insects subjected to foreign toxins (Kaufmann and Brown, 2008). The current study findings indicated considerable depletion in carbohydrate levels with both lethal concentrations in *Cx. pipiens* and *Cs.longiareolata*. These findings are in accordance with previous studies reporting a significant decrease in the levels of the carbohydrates in essential oil *Ruta graveolens* (LC_{50} and LC_{90}) treated *Cx. pipiens* larvae (Dris and Bouabida, 2020), this decrease was also observed in the 3rd instar larvae of *G. mellonella* after treatment with certain arthropod venoms (Tanani *et al.,* 2021). The larvae of *Culex quinquefasciatus* treated with the ethanolic extract of *Catharanthus roseus* revealed a significant decrease in glycogen and carbohydrate contents compared to the control (Shoba, 2018). Also, it is suggested that this carbohydrate depletion might be due to stresses of the tested arthropod venoms on proteins to be degraded into amino acids to take part in the TCA cycle of acetic acid. Subsequently, the carbohydrate metabolic functions to make up for the lower energy were altered under the stress of present toxic materials in the body of *galleria mellonella* (Tanani *et al.,* 2021).

Conflict of Interest

The authors declare that they have no conflict of interest.

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