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Ethanolic extracts of *Borago officinalis* L. affect growth, development and energy reserve profile in the mosquito *Culex pipiens*

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ABSTRACT

Ethanol extracts of leaves and flowers from Algerian *Borago officinalis* L. (Polemoniales: Boraginaceae) were evaluated against fourth instar larvae (L4) of *Culex pipiens* L. (Diptera: Culicidae). The larvicidal bioassay revealed that leaves extracts (LC_{50} = 2.49%) exhibited higher activity than flowers extracts (LC_{50} = 2.55%) against L4. Moreover, females were found more sensitive compared to males (sex-ratio was skewed towards males). On the other hand, the flower extract caused marked delayed effects on growth (decrease of female weight) and development (increase in duration of larval and pupal stages). The biochemical analyses showed a decrease in carbohydrate and lipid contents and an increase in protein levels in all tested stages (L4, pupa and adults male and female), with more significance in females.

Key words : *Borago officinalis, culex pipiens,* development, growth, toxicity.

INTRODUCTION

Because of their ability to transmit and spread diseases (malaria, dengue, Zika, chikungunya and yellow fever...) to humans, mosquitoes have been declared as their first enemy. For instance African Region showed the largest burden of malaria morbidity, with 200 million cases (92%) in 2017, followed by the South-East Asia Region (5%) and the Eastern Mediterranean Region (2%) (WHO, 2018a). Consequently, efforts to control mosquitoes were provided to prevent these epidemics (WHO, 2018b). Chemical control remains a major strategy for limiting vector density and mitigating pathogen transmission. However, the excessive use of synthetic insecticides generates a strong selection pressure, favouring the propagation of resistance alleles in natural populations (David *et al.,* 2018; Mastrantonio *et al.,* 2019). This phenomenon can disturb in a harmful way the natural ecosystems which disturbs the composition of the whole community (Weathered and Hammill, 2019). The importance of preventing insecticide resistance and the prospect of developing new molecules with the same efficiency and less damage seems become a necessity. Thus, new products such as insect growth disruptors (Hamaidia and Soltani, 2014; Hamaidia and Soltani, 2016; Hamaidia *et al.*, 2018; Hamaidia and Soltani, 2019) and plant derivative (Bouguerra *et al.*, 2017; Dris *et al.*, 2017; Barnawi *et al.*, 2019; Benelli *et al.*, 2019; Hung *et al.*, 2019; Martianasari and Hamid, 2019) have been tested.

Borago officinalis L. (Boraginaceae), commonly called borage, is an annual herbaceous plant. Its seeds, flowers and leaves have been used for culinary purposes (Miceli *et al.*, 2015), and especially medicinal by reducing the hepatotoxicity induced by radiation exposure (Rezk *et al.*, 2019) and improving the clinical symptoms of asthma (Mirsadraee *et al*., 2016). The plant has also shown its anti-inflammatory efficacy (Karimi *et al*., 2017), antimicrobial (Ali *et al.*, 2017) and and antioxydant activities (Khattab *et al.*, 2017). In another axis, the aqueous extract of *B. officinalis* flowers presented a potential inhibitor against the corrosion of mild steel (Al-Moubaraki, 2018). Experiments have been made on *B. officinalis* for culinary and medical aspects (Miceli *et al*., 2015; Mirsadraee *et al*., 2016). In contrast, few studies on pesticidal activity are done. A study on acaricide activity of flavonoid extract on

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Rhipicephalus sanguineus was only reported (El Haddad *et al*., 2018).

The objective of the current study was to evaluate the larvicidal activity of the ethanolic extracts of dried flowers and leaves of *B. officinalis* against the fourth instar larvae (L4) of *Culex pipiens* L. (Diptera: Culicidae), the most abundant mosquito species in Souk Ahras region located in Northesat Algeria (Hamaidia *et al*., 2016). Further, we examine the delayed effects of these extracts applied at two concentrations (LC_{30} and LC_{50}) on the growth and development (weight, development duration), and on the biochemical composition of different instars (L4, pupae and adults). Finally, the sex ratio has been taken into consideration.

MATERIALS AND METHODS

Mosquito rearing : The species *Cx. pipiens* was obtained from a stock colony of the Laboratory of Applied Animal Biology (Badji Mokhtar University, Annaba, Algeria). Larvae were fed with a mixture of biscuits-dry yeast (75 : 25 by weight) at 25 ± 2°C and 14 L : 10 D in Pyrex storage jars (80 × 100 mm) containing 150 ml of tap water as described by Rehimi and Soltani (1999). Three days after emergence, female adults were artificially fed with a blood meal (Hamaidia *et al*., 2018).

Ethanolic extract preparation : The entire plant of *B. officinalis* was collected in February 2019 in Souk-Ahras region (36°17′11″ N - 7°57′03″ E, Northeast Algeria). Then, flowers and leaves were separated, washed with tap water and rinsed with distilled water. Each component was then left to air-dry naturally at room temperature in laboratory for 5 days. At the end of this step, they are ground to a fine powder and the ethanolic extracts are prepared (Llorera *et al*., 2014). A powder sample (50 g) from each part of the plant (flowers and leaves) was extracted by cold maceration into 250 ml of ethanol and left for 24 h at room temperature. After filtration (Whatman paper No. 1), the filtrate is used as stock solution.

Treatment protocol : From the stock solutions, final concentrations ranged between 1.5 and 3.5% (V/V) were prepared for each ethanolic extract of *B. officinalis* and four replicates were made for each bioassay in the presence of 25 fourth instar larvae as recommended by WHO (2005). Two controls with the same number of larvae were performed simultaneously, the positive control which included ethanol and the negative control which consisted of tap water and food only. After 24 h of exposure, the dead larvae (unable to respond to tactile stimuli) were counted. The percentages of corrected (Abbott, 1925) and data subjected to probit analysis (Finney, 1971). The lethal concentrations were calculated with their respective confidence intervals (Swaroop *et al*., 1966).

Weight determination : After 24 h of treatment of the fourth instar larvae of *Cx. pipiens* with both ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}), the surviving individuals were rinsed with clean water, transferred to new cups. Samples of 10 individuals from each newly emerged stage (L4, pupa and adult male and female) were weighed and stored in an eppendorf tube containing 1ml of trichloroacetic acid (20%) and then subjected to biochemical analyses. The entire bioassay was repeated three times under similar conditions.

Development duration : The effects of ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}) on the duration of post-embryonic stages of *Cx. pipiens* were determined. Treated fourth instar larvae were transferred to white plastic cups containing 150 ml of tap water and food at the density of 10 larvae / cup. L4 were periodically observed at hourly intervals until pupation, indicating larval duration. Pupae development was checked until adult emergence which indicated pupal duration.

Sex ratio : After laying, the egg rafts were raised separately. L4 from the same raft were placed in 100 ml of an appropriate concentration of different ethanolic extracts (LC_{30} and LC_{50}) and maintained under bioassay conditions until adult emergence. The sex ratio was determined by counting males and females from the same raft. Each treatment was repeated three times with three rafts per repetition.

Biochemical procedure : Proteins, carbohydrates and lipids were extracted (Shibko *et al*., 1966) and quantified as described by Hamaidia *et al*. (2018). Samples (n = 10; fourth instar larvae 24 h, pupae and adults < 3 h) were extracted in 1 ml of trichloroacetic acid (20%). Proteins were quantified with coomassie brilliant blue (G 250; Merck, Germany) using bovine serum albumin (Sigma, St Louis, Missouri) as standard (Bradford, 1976). Carbohydrates were measured using anthrone reagent and glucose as standard (Duchateau and Florkin, 1958). Lipids were determined by the vanillin

method (Goldsworthy *et al*., 1972) and the table oil Afia was used as a standard. The results were expressed in µg/mg based on three replicates.

Statistical analysis : was performed using MINITAB software (version 16, PA State College, USA). The significance between the different series was tested using one-way analysis of variance (ANOVA) followed by a post-hoc honestly significant difference (HSD) Tukey's test and the level of significance considered is $p \leq 0.05$. The number of individuals tested per series is given with the results.

RESULTS AND DISCUSSION

Mosquitoes are harmful insects and major vectors of several life-threatening diseases (Omodior *et al*., 2018; Tandina *et al*., 2018). Vector borne diseases control programs have critical challenges, including the prevalence of mosquito resistance to synthetic insecticides and new threats of arboviruses that cause epidemics, such as chikungunya and Zika virus (Benelli and Mehlhorn, 2016), especially the high operational costs and toxic effects on human health (Naqqash et al., 2016). To solve all these challenges, new control strategies are an ecological necessity. The plant essential oils have demonstrated an optimal potential for insecticidal activity against several species (Dris *et al*., 2017; Bouguerra *et al*., 2019). In addition, several botanical extracts have been tested as mosquitoes larvicides (Hung *et al*., 2018), adulticides (Chansang *et al*., 2018; Martianasari and Hamid, 2019) or repellent (Lee, 2018). Recently, several studies have confirmed the antioxidant, anti-fungal, anti-inflammatory activities of ethanolic extracts of *B. officinalis* (Neagu *et al*., 2018), on the other hand, its insecticidal activity was studied only by testing the flavonoid extract of this plant against *R. sanguineus* (dog tick) which revealed a decrease in oviposition and hatching rate of eggs and significant toxicity for newly hatched larvae (El haddad *et al*., 2018).

The first part of the present study was devoted to the evaluation of toxicity to estimate lethal concentrations (CL_{30} and LC_{50}) of *B. officinalis* leaves and flowers against L4 of *Cx. pipiens*. The ethanolic extracts were used at different final concentrations ranging between 1.5 and 3% for on newly fourth instar larvae (< 8 h) of *Cx. pipiens*. The corrected mortalities were varying from 4.00 to 85.00% and 47.20 to 94.07% respectively in a dose-response relationship ($p < 0.05$). Based on the regression equations, lethal concentrations (LC_{30} and LC_{50}) were calculated (Table 1). Khurm *et al*. (2016) showed that the crude dichloromethane extract of *Heliotropium strigosum* (Boraginaceae) had a moderate insecticidal activity (40% inhibition) against *Rhozopertha dominica* and a low activity (20% inhibition) against *Sitophilus oryzae*, while the methanolic extract did not show any significant activity. Extracts of petroleum ether, ethyl acetate and methanol from leaves of *Heliotropium indicum* (Boraginaceae) revealed an insecticidal activity anti-feeding against *Helopeltis theivora* (Dolui *et al*., 2010).

Then, several delayed effects were considered on different stages of development. According to results mentioned in Table 2, the weight of the surviving larvae, pupae and adult males of the treatment was not affected, for both applied concentrations (LC_{30} and LC_{50}); either leaves or flowers as compared to the control ($p > 0.05$). However, the weight of the females was significantly reduced following the application of flower extract only (Control vs CL_{30} F: $F_{1, 5} = 178$, $p < 0.001$, Control vs $CL_{50\ F}$: $F_{1, 5} = 81.31$, $p < 0.001$), while the leaves extract had no significant effects as compared to control series ($p > 0.05$). The results of the effect of the different ethanolic extracts of the leaves and flowers of *B. officinalis* on the development duration of L4 and pupa are presented in Fig. 1. The development durations were significantly elongated ($p < 0.001$) for all tested concentrations (LC_{30} and LC_{50}). L4 : (Control vs $CL_{30\ L}$: $F_{1, 206} = 28.13$; Control vs $CL_{50\ L}$: $F_{1, 181} = 82.44$ with dose-response relationship; $CL_{30\ L}$ vs $CL_{50\ L}$: $F_{1, 291} = 70.46$, $p < 0.001$) (Control vs $CL_{30\ F}$: $F_{1, 230} = 83.19$; Control vs $CL_{50\ F}$: $F_{1, 197}$

Table 1. Efficacy of ethanolic extracts of *B. officinalis* applied to 4th instar larvae of *Cx. pipiens* after 24 h (n = 4 repetitions each containing 25 individuals).

Extract	LC_{30} (%)	LC_{50} (%)	Slope	P	Regression equation	R^2
Leaves	2.17	2.49	2.59	$p < 0.05$	Y = 8.740X + 1.540	0.928
Flowers	2.40	2.55	2.23	$p < 0.05$	Y = 20.47X - 3.321	0.955

Table 2. Effect of ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}) of leaves (L) and flowers (F) on the weight (mg) of different stages (L4 aged 24 h, Pupa and adults just after emergence) of *Cx. pipiens* (m ± SD, n = 3, 10 individuals/pool).

Series	L4	Pupa	Male adult	Female adult
Control	2.05 ± 0.55a	4.97 ± 0.41a	1.90 ± 0.24a	3.67 ± 0.07a
$LC_{30\ L}$	2.44 ± 0.29a	5.03 ± 0.42a	1.95 ± 0.16a	3.07 ± 0.40a
$LC_{50\ L}$	2.47 ± 0.29a	4.84 ± 1.22a	1.76 ± 0.21a	3.48 ± 0.28a
$LC_{30\ F}$	2.23 ± 0.05a	4.99 ± 0.36a	2.03 ± 0.21a	3.07 ± 0.01b
$LC_{50\ F}$	2.09 ± 0.71a	5.92 ± 0.54a	1.97 ± 0.09a	3.19 ± 0.05b

For each stage, values followed by the same letter are not significantly different at $p > 0.05$.

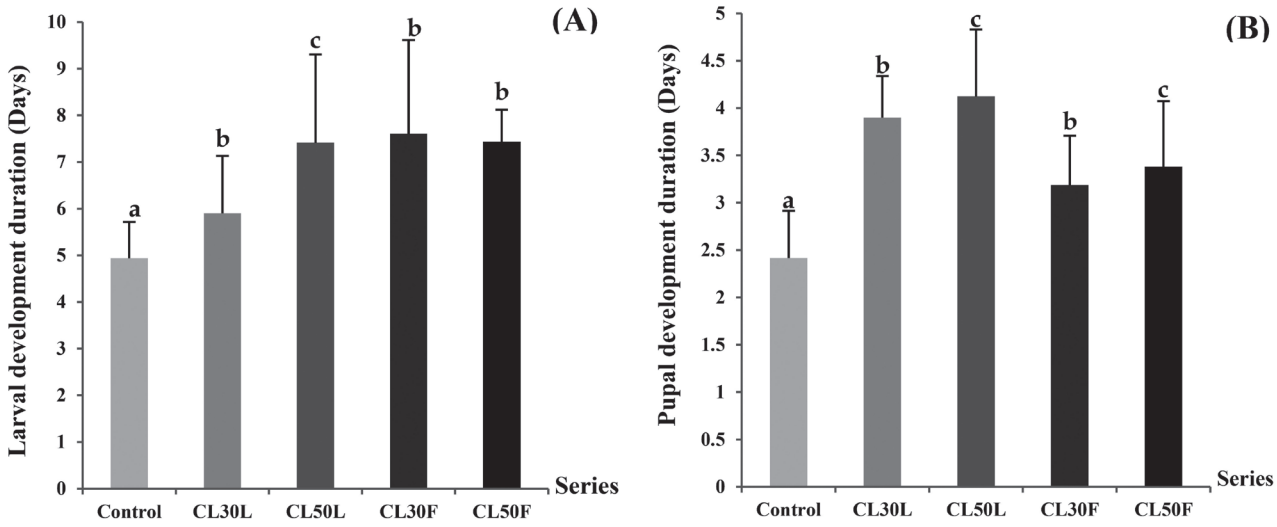

Fig. 1. Effects of ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}) of leaves (L) and flowers (F) on development duration of fourth instar larvae (A) and pupa (B) of *Cx. pipiens* (m ± SD, n = 175-331).

= 465.55 without dose-response relationship $CL_{30\ F}$ vs $CL_{50\ F}$: $F_{1, 331} = 0.94$, $p > 0.05$). Pupa : (Control vs $CL_{30\ L}$: $F_{1, 180} = 364.15$; Control vs $CL_{50\ L}$: $F_{1, 175} = 230.76$ with dose-response relationship; $CL_{30\ L}$ vs $CL_{50\ L}$: $F_{1, 261} = 7.48$, $p = 0.006$) (Control vs $CL_{30\ F}$: $F_{1, 219} = 84.14$; Control vs $CL_{50\ F}$: $F_{1, 196} = 79.68$ with dose-response relationship; $CL_{30\ F}$ vs $CL_{50\ F}$: $F_{1, 321} = 8.35$, $p = 0.004$).

The results revealed larvicidal activity of the ethanolic leaves extract (LC_{50} = 2.49%) greater than that of the flowers extract (LC_{50} = 2.55%) of *B. officinalis*. According to the results presented in Fig. 2, only the sex ratio of the treated series with *B. officinalis* leaves extract, by both tested concentrations, was modified in favour of males (males vs females: $LC_{30\ L}$: $F_{1, 5} = 10.67$; $p = 0.030$ [M = 65.90% /F = 34.09% : 2/1]; $LC_{50\ L}$: $F_{1, 5} = 11.63$; $p = 0.026$ [M = 64.10% /F = 35.89% : 2/1]) as compared with control series (males vs females: $F_{1, 5} = 4.78$, $p = 0.093$ [M = 51.15% /F = 48.84% : 1/1]). According to data, females appeared to be more sensitive to treatment than males (sex ratio was biased to males 2/1). The duration of development of L4 as well as pupae were prolonged significantly after application of all extracts with a concentration-response relationship. Female weight was affected by both concentrations of the ethanolic extract of the flowers. Growth disruptor compounds (natural or synthetic) interfered with the endocrine system of organisms; therefore it deregulated physiological functions hormonally controlled (Combarnous, 2017). Also, an increase (Hamaidia and Soltani, 2014) or a reduction of the development duration (Hamaidia *et al*., 2018) were considered as a sign of a growth disruption. By analyzing our results, probable “growth disruptor effect” could be attributed to the ethanolic extracts from *B. officinalis*. Susceptibility of females as compared to males (in

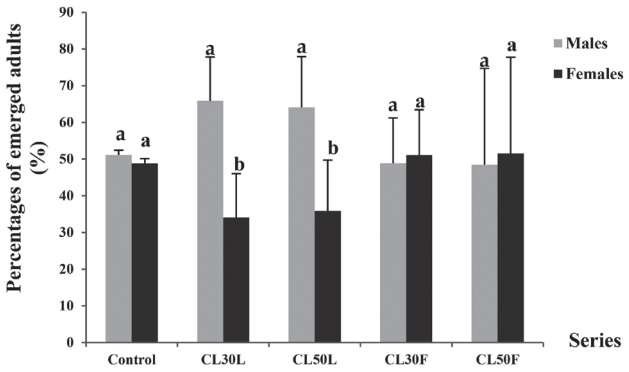

Fig. 2. Effects of ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}) of leaves (L) and flowers (F) on sex ratio of *Cx. pipiens* (m ± SD, n = 3 rafts).

regarding sex ratio, weight and differential mortality between both sexes) reinforces this hypothesis. Diterpene from *Lindera erythrocarpa* and *Solidago serotina* showed effectiveness as juvenile hormone antagonist against mosquitoes causing reduction in the expression of Met target genes and retardation of follicle development in mosquito ovaries (Lee *et al*., 2015). Methanol extract of *Sargassum binderi* exhibited a strong prolongation of larval period of *Aedes aegypti* (Yu *et al*., 2015). *L. erythrocarpa* also disrupted the development of larvae and pupae in *Drosophila melanogaster* (Shin *et al*., 2018). Moreover, diethyl ether extract of leaves from *Nerium oleander* (Apocynaceae) decreased the larval and pupal duration and a significant lethality in both male and female adults of *Cx. pipiens* (El-Sayed and El-Bassiony, 2015).

Based on the reference equations the main biochemical constituents (carbohydrates, proteins and lipids) were quantified during the tested stages (L4, pupae and adult males and females). As shown in Table 3, whole body carbohydrate contents of fourth instar larvae and males were unaffected by the two tested concentrations of both ethanolic extracts of *B. officinalis* ($p > 0.05$). On the other hand, a significant decrease in this parameter was revealed in pupae after application of the LC_{30} ($F_{1, 4}$ = 24.29, p = 0.007) and LC_{50} ($F_{1, 4}$ = 19.72, p = 0.011) of the leaves extract, and the higher concentration of flowers only ($LC_{50\ F}$: $F1_{, 4}$ = 19.94, p = 0.011) compared to controls. In addition, the females were more sensitive to the extracts (Control vs $LC_{50\ L}$: $F_{1, 4}$ = 28.83, p = 0.005, Control vs $CL_{30\ F}$: $F_{1, 4}$ = 10.56, p = 0.031, Control vs $LC_{50\ F}$: $F_{1, 4}$ = 112.41; p = 0.0004). Concerning the total proteins (Table 3) it can be seen that the two pre-imaginal tested stages (L4 and pupa) did not shown changes in total protein contents compared to control ($p > 0.05$). In the adult stage, the whole body protein

Table 3. Effect of ethanolic extracts of *B. officinalis* (LC_{30} and LC_{50}) of leaves (L) and flowers (F) on metabolites contents (µg/mg) of different stages of *Cx. pipiens* (m ± SD; n= 3 repeats each with 10 individuals).

Constituents	Series	L4	Pupae	Male	Female
Carbohydrates	Control	11.585 ± 3.725a	5.754 ± 1.401a	5.315 ± 2.020a	6.525 ± 0.534a
	$LC_{30\ L}$	7.680 ± 0.457a	1.491 ± 0.529b	5.398 ± 1.729a	8.302 ± 1.422a
	$LC_{50\ L}$	10.436 ± 2.281a	1.995 ± 0.431c	2.319 ± 1.432a	4.265 ± 0.495b
	$LC_{30\ F}$	8.514 ± 1.763a	4.246 ± 1.368a	4.071 ± 0.979a	3.217 ± 1.679c
	$LC_{50\ F}$	5.134 ± 3.878a	2.012 ± 0.377c	2.482 ± 1.683a	1.513 ± 0.620d
Proteins	Control	0.136 ± 0.041a	0.052 ± 0.016a	0.062 ± 0.009a	0.052 ± 0.008a
	$LC_{30\ L}$	0.144 ± 0.021a	0.061 ± 0.019a	0.052 ± 0.017a	0.058 ± 0.009a
	$LC_{50\ L}$	0.131 ± 0.050a	0.066 ± 0.024a	0.047 ± 0.009a	0.078 ± 0.010b
	$LC_{30\ F}$	0.148 ± 0.011a	0.024 ± 0.008a	0.087 ± 0.009b	0.111 ± 0.006c
	$LC_{50\ F}$	0.178 ± 0.051a	0.048 ± 0.016a	0.084 ± 0.005b	0.124 ± 0.010c
Lipids	Control	2.427 ± 0.764a	1.194 ± 0.282a	1.178 ± 0.416a	1.364 ± 0.108a
	$LC_{30\ L}$	1.625 ± 0.101a	0.341 ± 0.108b	1.191 ± 0.346a	1.731 ± 0.289a
	$LC_{50\ L}$	2.175 ± 0.447a	0.445 ± 0.097c	0.588 ± 0.297a	0.915 ± 0.103b
	$LC_{30\ F}$	1.799 ± 0.350a	0.892 ± 0.273a	0.921 ± 0.206a	0.713 ± 0.335c
	$LC_{50\ F}$	1.138 ± 0.758a	0.439 ± 0.077c	0.429 ± 0.036b	0.370 ± 0.124d

For each stage, values followed by the same letter are not significantly different at $p > 0.05$.

content of males was significantly increased by the flower extract with both concentrations in a dose-response relationship (control vs $CL_{30\ F}$: $F_{1, 4}$ = 9.70, p = 0.035; Control vs $LC_{50\ F}$: $F_{1, 4}$ = 11.94, p = 0.025). For females, the highest concentration of leaf extract (Control vs $LC_{50\ L}$: $F_{1, 4}$ = 10.87, p = 0.029) as well as the two concentrations of flowers (Control vs $CL_{30\ F}$: $F_{1, 4}$ = 97.12, p = 0.0005; Control vs $LC_{50\ F}$: $F_{1, 4}$ = 90.95; p = 0.0006) also caused an increase in this parameter. Regarding the total lipid contents in the whole body of different stages of development of *Cx. pipiens*, this parameter was not affected by treatment in fourth instar larvae (p > 0.05). In pupae, both leaves concentrations caused a significant decrease in total lipid contents (control vs $CL_{30\ L}$: $F_{1, 4}$ = 23.82, p = 0.008, control vs $LC_{50\ L}$: $F_{1, 4}$ = 18.85, p = 0.012). In contrast, the highest concentration of flower extracts was significant compared to the control (Control vs $LC_{50\ F}$: $F_{1, 4}$ = 19.95, p = 0.011). In males, this rate was negatively affected with the highest tested concentration of flowers (control vs $LC_{50\ F}$: $F_{1, 4}$ = 9.60, p = 0.036). Finally, in females, the LC_{50} of the leaves extracts ($F_{1, 4}$ = 26.88, p = 0.006), the CL_{30} and the LC_{50} of the flowers extracts (control vs $CL_{30\ F}$: $F_{1, 4}$ = 10.18, p = 0.033, control vs $LC_{50\ F}$: $F_{1, 4}$ = 109.08, p = 0.0004) caused a decrease in lipid contents. For the whole body biochemical composition of L4, pupae and adult male and female, metabolic disruptions have been observed. The decrease in total carbohydrate and lipid contents can be attributed to mobilization of reserves to cope with stress caused by treatment. In addition, there has been an increase in total protein which may be due to an increase in synthesis of detoxification enzymes. *Cx. pipiens* responded by metabolic changes to face stress induced by *B. officinalis*. Based on the results of biochemical status of *Culex quinquifasciatus* larvae treated by *Catharanthus roseus* ethanolic leaves extracts, significant depletion of glycogen and carbohydrate levels was indicated when compared to control (Shoba, 2018). It was revealed that phenolic fraction of *Ziziphus jujuba* (Rhamnaceae) decreased adult life span accompanied by decrease in carbohydrate and lipid levels in *Aedes aegypti*, (Devi and Bora, 2017). Petroleum ether extract of *Artemisia annua* reduced the total carbohydrates, lipid and protein levels in *Culex quinquefasciatus* larvae (Sharma *et al*., 2011). In *Cx. quinquefasciatus* larvae, total protein and glycogen levels were declined by apigenin extracted from *Jatropha gossypifolia* leaves, while an enhancement of free amino acid level was noted (Johnson and Singh, 2017). Depending on all of the above, the impacting factors of extracts on metabolic activity of larvae are species specific (Shoba, 2018).

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