Toxicological Effects of *Lagocephalus scleratus* fish extracts against *Culex pipiens* (Diptera: Culicidae)

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Abstract: Three extracts of Skin (SE), Muscle (ME) and Viscera (VE) of *Lagocephalus scleratus* were evaluated against immature and mature stages of *Culex pipiens* to assess the toxicity, LC_{50}, fecundity, egg hatchability and some biochemical parameters. The survival potential of larval stage was highly affected by the treatment with the tested extracts. On the basis of LC_{50}, ME was the most toxic one against the larval stage followed by SE and VE. The late toxicity of fish extracts tested on the adult females resulted from larvae treated with the LC_{50} of each extract decreased the number of eggs laid by female. The fecundity recorded 88.3±2.9, 136.7±7.6 and 150±5 eggs/♀ for females resulted from larvae treated with the LC_{50} of ME, SE and VE; respectively, compared to 171.7±7.6 eggs/♀ for control females. The hatchability percent of eggs laid by females treated with the LC_{50} of ME, SE and VE was decreased to 33.9, 51.3 and 60%; respectively, compared to 90.3% for eggs laid by untreated females. A marked decrease in total carbohydrate, lipid and protein contents in the whole body of males and females, *C. pipiens* resulted from larvae treated with the LC_{50} of ME, SE and VE were observed. It is clear from the results obtained in this study that puffer toxins are effective in the mosquito control.
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1. Introduction:

Mosquitoes top all the insect-vectors in transmission of serious zoonotic diseases worldwide. *Anopheles stephensi*, *Aedes aegypti* and *Culex pipiens* are the vector mosquitoes of malaria, dengue and lymphatic filariasis, respectively. Over two billion people in tropical countries are at risk from mosquito borne diseases and the search for effective vaccines against these diseases is still in progress (WHO, 2008). In Egypt, *C. pipiens* is widely distributed (Mikhail *et al.*, 2009), which has natural and artificial breeding, sites in the endemic and non-endemic villages (Harb *et al.*, 1993). Apart from filariasis, Culicini, mainly *C. pipiens* transmit Sindbis virus (Wilson, 1991), West Nile Virus, Rift Valley Fever and Dog Heartworm (El-Bahnasawy *et al.*, 2013).

The unplanned use of chemical insecticides during the past few decades to control insect pests have resulted in serious consequences such as insect resistance, mammalian toxicity, bioaccumulation through food chains, environmental contamination and risk for human health (Klein, 1976). This necessitates the search for new sources for insect control agents.

The marine environment represents a treasure of useful products a waiting discovery for the treatment of fungal, parasitic, bacterial and viral diseases and also as insecticidal products. A small number of marine plants, animals and microbes have already yielded more than 12000 novel chemicals, with hundreds of new compounds still being discovered every year (Donia and Hamann, 2003).

Secondary metabolites of marine organisms differ from that of terrestrial organisms. Bioactive compounds isolated from marine organisms exhibits various biological activities such as anti-cancer, anti-inflammatory, antifungal, antimicrobial and mosquito larvicidal properties (Gul & Hamann, 2005; Venkateswara Rao *et al*., 1995, 2008). The extracts of marine sponges *Clathria longitoxa*, *Callyspongia diffusa*, *Haliclona pigmentifera*, *Sigmadocia carnosa* and *Denrilla nigra* showed significant insecticidal activity against mosquito larvae and agricultural pests (Baby *et al*., 2010). The larvicidal potential of prawn *Nematopalaemon tenuipes* and sea cucumber *Holothuria scabra* extracts have been reported (Narsinh *et al*., 2004). In the present paper we report the toxicological effects of different *L. scleratus* puffer fish extracts (PFEs) against *C. pipiens.*

2. Materials and Methods.

2-1- Origin and laboratory maintenance of the mosquito colony:

Mosquitoes used in this study were *Culex pipiens* L., they were collected from Abu-Rawash, Giza governorate, then were reared for several generations, in the insectary of medical entomology at the department of zoology, faculty of science, under controlled conditions at temperature of 27±2 °C, relative humidity 70±10% and 12-12 light-dark

regime. Adult mosquitoes were daily provided with sponge pieces soaked in 10% sucrose solution for a period of 3-4 days after emergence. After this period the females were allowed to take a blood meal from a pigeon host, which is necessary for laying eggs. The resulting egg rafts picked up and transferred into plastic pans containing 3 liters of tap water left for 24 h. The hatching larvae were provided daily with fish food as a diet. This diet was found to be the most preferable food for the larval development and a well female fecundity, (Kasap and Demirhan, 1992).

2-2- Collection of puffer fish:

Puffer fish, *Lagocephalus scleratus* was collected from different fishing areas of Alexandria coast in Egypt. All the samples were transported in iceboxes to the laboratory at faculty of science, Cairo and subsequently kept frozen at -20ºC until use.

2-3- Preparation of the crude extract:

The puffer fish identification was done based on the morphological characteristics, then it was partially thawed and excised the tissues of muscle and skin and collected separately in addition to the viscera. Toxins was extracted from each tissue according to Helbig and Luckas (2010) with slight modifications, each tissue was minced and a small portion (1g) was extracted with 3 ml of 0.03M acetic acid using an ultrasonic probe (OMNI-Ruptor 4000, Georgia, USA) for 1 min. the homogenate then centrifuged at 5000 rpm for 15 min (Eppendroff 5430, Hamburg, Germany) and subsequently the supernatant was collected and transferred to a volumetric flask of 10 ml. The sample extract then filtered with 0.45 µm Nylon membrane filter.

2-4- Experimental bioassay:

In order to study the toxicity of these PFEs, different range of concentrations of each extract was used. The 2nd instar larvae were collected from the established colony and placed in plastic cup containing 250 ml of the extract solution as recommended by (WHO). Control larvae were placed in cups contained 250 ml dechlorinated tap water (25 of 2nd instar larvae/cup). At least three replicates were used in each experiment. All plastic cups were incubated under controlled conditions at temperature of 27±2 °C, relative humidity 70±10% and 12-12 light-dark regime. The following biological aspects were used to evaluate the effect of the different PFEs on *C. pipiens*.

2-4-1- Larvicidal activity:

Larval mortality was recorded daily and dead larvae removed until adult emergence. Mortality of the larvae was indicated by a failure to respond to mechanical stimulation (Williams *et al.* 1986). Larval mortality percent was estimated by using the following equation: Larval mortality % = A–B/A ×100 (Briggs, 1960): Where: A= number of tested larvae. B= number of tested pupae.

2-4-2- Female fecundity:

The adult females that succeeded to emerge from larvae treated with the LC_{50} of different PFEs were collected and transferred with normal adult males obtained from the colony by using an electric aspirator recommended by (WHO), and fed with 10% sugar solution for three days. Then, the adult males and females leaved one day without sugar solution. At day five, the starved females were allowed to take a blood meal from a pigeon and allowed to lay egg rafts on clean water. The number of eggs/raft was counted by using binocular microscope and the mean value was taken.

2-4-3- Egg hatchability:

The eggs of females resulted from larvae treated with the LC_{50} of different PFEs were counted by using a binocular microscope. The eggs were sorted into two categories: hatched and non-hatched eggs according to the method used by Hassan *et al*. (1996). The Egg hatchability was calculated by using the following equation: Egg-hatchability % = A/B×100, Where: A = total no. of hatched eggs. B = total no. of eggs laid.

2-5- Biochemical studies:

The adult males and females resulted from *C. pipiens* larvae treated with the LC_{50} of different PFEs were collected daily, weighed and kept under freezing condition at 4 ^{o}C until the biochemical determinations. For the determination of the total carbohydrate, lipid and protein, adults were homogenized in saline solution (40 adults/1ml saline solution) using fine electric homogenizers, tissue grinder for 2 min. Homogenates were centrifuged at 4000 r.p.m. for 15 min. at 2 ^{o}C in a refrigerated centrifuge. The supernatant was used directly or stored at 4 ^{o}C until biochemical determination.

2-5-1- Determination of the total carbohydrate contents:

The total carbohydrate contents of the whole adult body were determined according to Singh and Sinha (1977).

2-5-2- Determination of the total lipid contents:

The total lipid contents were determined by colorimetric method of (Frings *et al*., 1972). A sample of whole body extract was heated with concentrated sulphuric acid and the mixture was then reacted with phosphoric acid-vanilline reagent to give red to purple color. The intensity of color was measured by photoelectric colorimeter (Carlziss).

2-5-3- Determination of the total protein contents:

Total protein contents were determined using the method of (Lowery *et al*., 1951). Protein reacts with the folin ciocalteau reagent to give a blue colored complex. The color formed is due to reaction of alkaline copper with the protein. The intensity of the

color was measured photometrically by using a Bausch and Lomb spectrophotometer 710 of 750 nm.

2-6- Statistical analysis:

The statistical analysis of the obtained data was done according to Armitage (1974) and Lentner *et al*. (1982). The analysis was revised and graphics were drown by Excel for windows program Microsoft office 2010. The obtained data were assessed by calculation of the mean M, standard deviation SD. The LC_{50} was calculated using multiple linear regressions (Finney, 1971).

3. Results.

3-1- Biological activity of PFEs against *Culex pipiens*.

3-1-1- Toxicity.

A- Skin extract:

The mortality percentages of *C. pipiens* larvae as influenced by different concentrations of *Lagocephalus scleratus* SE are given in table (1). The obtained data indicated that there was a positive correlation between the concentration of the SE and the mortality percent i.e. the increase of SE concentration led to the increase of larval mortality percent. The larval mortality percent increased gradually from 12.0% at the concentration of 2000 ppm to 97.3% at the concentration of 10000 ppm. The larval mortality percent among the control group was 1.3%. The calculated LC_{50} from the different mortality percentages recorded 5211.2 ppm.

Table (1): Effect of different concentrations of SE of *L. scleratus* on larval mortality of *C. pipiens*.

Conc.	Tested Larvae	Mean larval Mortality	Mortality %	Lc $_{(50)}$ ppm
10000	25	24.3	97.3	5211.2
8000	25	22.3	89.3	
6000	25	14.7	58.7	
4000	25	10.3	41.3	
2000	25	3.0	12.0	
Control	25	0.3	1.3	

B- Muscle extract:

The data given in table (2) showed the effect of different concentrations of *L. scleratus* ME on the larval mortality percentages. There was a positive correlation between the concentration of ME and the mortality percent. The larval mortality percent increased from 9.3% at the concentration of 1000 ppm to 94.7% at the concentration of 8000 ppm. The larval mortality percent among the control group was 1.3%. The LC_{50} as calculated from the different mortality percentages recorded 3715.3 ppm.

Table (2): Effect of different concentrations of ME of *L. scleratus* on larval mortality of *C. pipiens*.

Conc.	**Tested Larvae**	**Mean larval Mortality**	**Mortality %**	**Lc $_{(50)}$ ppm**
8000	25	23.7	94.7	3715.3
6000	25	20.7	82.7	
4000	25	15.3	61.3	
2000	25	9.3	37.3	
1000	25	2.3	9.3	
Control	25	0.3	1.3	

C- Viscera extract:

Data given in table (3) showed the larval mortality percentages among the larvae treated with different concentrations of VE as well as the untreated ones. The results indicated that, the larval mortality percent increased as the concentration of VE increased. The larval mortality percent increased from 9.3% at the concentration of 1000 ppm to 93.3% at the concentration of 12000 ppm. The larval mortality percent was 2.7 % among the control group. The calculated LC_{50} from the different mortality percentages recorded 5578.9 ppm.

Table (3): Effect of different concentrations of VE of *L. scleratus* on larval mortality of *C. pipiens*.

Conc.	Tested Larvae	Mean larval Mortality	Mortality %	Lc $_{(50)}$ ppm
12000	25	23.3	93.3	5578.9
9000	25	21.0	84.0	
6000	25	13.3	53.3	
3000	25	10.0	40.0	
1000	25	2.3	9.3	
Control	25	0.67	2.7	

From the aforementioned results it is obvious that the toxicity values of the tested PFEs based on LC_{50} (Table 4) may be arranged in descending order as follows: ME > SE > VE.

Table (4): Toxicity of different PFEs of *L. scleratus* against larvae of *C. pipiens*.

Extracts	LC_{50} (ppm)	Slope (b)	Correlation Coefficient (r)
Skin	5211.2	2.5778	0.0091
Muscle	3715.3	13.962	0.0097
Viscera	5578.9	14.853	0.0063

3-1-2- Fecundity.

The number of eggs laid per female (fecundity) for *C. pipiens* females resulted from treated larvae with the LC_{50} of SE, ME and VE and others (untreated) is given in table (5). As shown from the results there was a marked decrease of eggs laid by females resulted from larvae treated with the LC_{50} of ME, SE and VE, where the fecundity was 88.3±2.9, 136.7±7.6 and 150±5 eggs/♀; respectively, compared to 171.7±7.6 eggs/♀ for the untreated females (control).

Table (5): Fecundity and egg hatchability of *C. pipiens* affected by treatment with the LC_{50} of different PFEs of *L. scleratus*.

Extracts	LC_{50} (ppm)	No. of tested Females	No. of eggs laid		No. of Egg hatched	
			Total	Mean± S.D.	Total	%
Skin	5211.2	25	3417	136.7±7.6	1750	51.3
Muscle	3715.3	25	2207	88.3±2.9	750	33.9
Viscera	5578.9	25	3750	150.0±5.0	2250	60.0
Control	--	25	4292	171.7±7.6	3875	90.3

3-1-3- Egg hatchability.

The hatchability percent of eggs laid by *C. pipiens* females resulted from larvae treated with the LC_{50} of different PFEs of *L. scleratus*, and the other resulted from untreated larvae is also given in table (5). The results indicated that PFEs used decreased the hatchability percent of eggs laid by females resulted from treated larvae as compared with the control. The hatchability percent recorded was 33.9, 51.3 and 60% for eggs laid by females treated with the LC_{50} of ME, SE and VE; respectively, compared to 90.3% for untreated larvae.

3-2- Effect of PFEs on some biochemical parameters in *C. pipiens*.

3-2-1- Total carbohydrate contents:

Data given in table (6) showed the changes in the total carbohydrate contents in the homogenate of the whole body of *C. pipiens* adults resulted from larvae treated with the LC_{50} of different PFEs as well as the control group. The results indicated a marked decrease in total carbohydrate contents in the whole body of females resulted from larvae treated with the LC_{50} of SE, ME and VE, The total carbohydrate recorded 0.08, 0.07 and 0.09 mg/ml; respectively, compared to 0.14 mg/ml for untreated females. On the other hand, the change in total carbohydrate contents in males resulted from larvae treated with the LC_{50} of different PFEs exhibited only slight decrease. It recorded 0.11, 0.106 and 0.113 mg/ml for SE, ME and VE; respectively, compared to 0.12 mg/ml for control males.

Table (6): Changes in the total Carbohydrate, Lipid and Protein contents of *C. pipiens* resulted from larvae treated

with the LC_{50} of *L. scleratus* PFEs.

Treatments	LC_{50}	Carbohydrate (mg/ml)	Change %	Lipid (mg/ml)	Change %	Protein (mg/ml)	Change %
Skin (male)	5211.2	0.11	-8.3	0.11	-42.1	0.25	-16.7
Skin (female)	5211.2	0.08	-42.9	0.47	-30.9	0.38	-7.3
Muscle (male)	3715.3	0.106	-11.7	0.098	-48.4	0.26	-13.3
Muscle (female)	3715.3	0.07	-50.0	0.47	-30.9	0.37	-9.8
Viscera (male)	5578.9	0.113	5.8	0.13	-31.6	0.26	-13.3
Viscera (female)	5578.9	0.09	-35.7	0.55	-19.1	0.4	-2.4
Control (male)	--	0.12	--	0.19	--	0.3	--
Control (female)	--	0.14	--	0.68	--	0.41	--

3-2-2- Total Lipid contents:

As shown from the results given in table (6) a marked decrease in total lipid contents in males and females resulted from larvae treated with LC_{50} of different PFEs was occurred, where in females it recorded 0.47, 0.47 and 0.55 mg/ml for SE, ME and VE; respectively, compared to 0.68 mg/ml for untreated females. In males the total lipid contents recorded 0.11, 0.098 and 0.13 mg/ml for SE, ME and VE; respectively, compared to 0.19 mg/ml for untreated males.

3-2-3- Total protein contents:

Data given in table (6) revealed that, the total protein contents in males and females resulted from larvae treated with LC_{50} of different PFEs was slightly reduced in each tested extract.

4. Discussion:

Majority of the mosquito control programs are targeting the immature stages of the mosquitoes as the principal breeding habitats are man-made and can be easily identified (Howard *et al.*, 2007). Phytochemicals and microbial metabolites are used as alternatives to conventional broad spectrum of synthetic insecticides. The toxins derived from the natural sources are biodegradable and less prone to the development of resistance, which makes them environmentally sound control agents. Animals have also been a source of some interesting compounds that can be used as drugs/insecticides.

Previous literature indicated that marine organisms possess maximum percentage of bioactive substances with novel biological properties than the molecules originated from terrestrial origin (Venkateswara-Rao *et al*., 1995; Gul & Hamann, 2005). Marine natural products provide a novel and rich source of chemical diversity that can contribute to design and development of new bioactive molecules. However not many reports on the mosquito larvicidal activity of marine natural products except for the extracts of sponges (Venkateswara-Rao *et al*., 1995, 2008) sea cucumber and prawn (Narsinh *et al*., 2004) are available.

Earlier reports showed that in most marine puffers, high concentrations of tetrodotoxin or TTX (toxin acts on the central and the peripheral nervous systems) are found with significant amounts in digestive tissue, muscles and skin (Fuchi *et al*., 1991; Noguchi & Arakawa, 2008). The TTX has the potential to serve as an anti-cancer drug by showing an inhibitory effect on the invasiveness of metastatic prostrate cancer (Prasad *et al*., 2004), to be developed as an anesthesia agent (Schwartz *et al*., 1998) and as a painkiller for chronic cancer pain (Narahashi, 2001). However, so far no work has been reported for the effect of this toxin on mosquito larvae and adults. This work has been initiated to exploit the possibility of using PFEs for mosquito larval control.

In the present study, different parts; skin, muscle and viscera were separated from *L. scleratus* and extracted with 1% acetic acid, cleared the sclero-proteins and removed the solvent under reduced pressure followed by neutralization. The material was screened against larvae and adults of mosquito species, *C. pipiens* as per standard procedure (WHO, 2005). The results showed that ME was more effective in killing the mosquito larvae than the SE and VE.

The results obtained revealed that the PFEs were found to exert biological effects on the larvae of *C. pipiens*. The survival potential of the larval stage was highly affected by PFEs tested. A concentration dependent mortality percent was obtained i.e. the larval mortality percent increased as the concentrations of extracts increased. However, the present data revealed that the toxicity of tested extracts against the larval stage varied from one extract to another.

On the basis of LC_{50}, ME was the most toxic extract against the larval stage followed by SE and VE. These results are in agreement with those obtained by (Samidurai and Mathew, 2013), where they reported high toxicity of ME followed SE of puffer fishes, *Arothron hispidus* and *L. inermis* against larvae of the mosquito vector, *Anopheles stephensi.* The mosquito larvicidal activity of PFEs are

comparable to that of prawn and sea cucumber extracts (Narsinh *et al*., 2004), where they found that puffer toxins effective in killing the larvae of mosquitoes at higher concentrations >1000ppm.

The present data have shown also that the LC_{50} was 5211.2 and 3715.3 ppm for SE and ME; respectively, against *C. pipiens* larvae. Meanwhile, (Samidurai and Mathew, 2013) reported that, the LC_{50} for SE and ME was (10817.8 and 7116.8 ppm) for puffer fish *Arothron hispidus* and it was (10283.04 and 6067.5 ppm) for puffer fish *L. inermis;* respectively, against the larvae of *Anopheles stephensi.*

Concerning the effect of PFEs on reproduction, reports on the toxic effects of PFEs on insect reproduction are rare. Samidurai, (2011) stated that the nereistoxin affecting processes such as reproduction and hatchability of *Anopheles stephensi, Aedes aegypti* and *C. quinquefasciatus.* The present study has shown that the delayed toxicity of PFEs on the adult females resulted from larvae treated with the LC_{50} of the tested extracts decreased significantly the number of eggs laid per female. The fecundity was 136.7±7.6, 88.3±2.9 and 150.0±5.0eggs/♀ for females resulted from larvae treated with the LC_{50} of SE, ME and VE; respectively, compared to 171.7±7.6 eggs/♀ for control females. The observed inhibition of hatching of eggs laid by females resulted from larvae treated with the LC_{50} of the PFEs tested as indicated in the present study was in agreement with Samidurai, (2011) using nereistoxin against three mosquito species.

Research on marine natural insecticidal products affects mosquito biochemistry has started only recently. Only scattered information on its effects on metabolism is available. Conclusion on how these products interfere with general metabolic pathways can probably be drawn from the determination of total protein, carbohydrate and lipid in the hemolymph or the target organ or even the whole body.

The results indicated a marked decrease in total carbohydrate contents in the whole body of females resulted from larvae treated with the LC_{50} of SE, ME and VE. On the other hand, the change in total carbohydrate contents in males resulted from larvae treated with the LC_{50} of different PFEs exhibited only slight decrease. A marked decrease in total lipid contents in males and females resulted from larvae treated with LC50 of different PFEs was occurred. Also, the total protein contents in males and females resulted from larvae treated with LC_{50} of different PFEs was slightly reduced in each tested extract.

The use of natural marine products is an alternative pest control method, which helps to minimize the usage of toxic pesticides and their deleterious effects on insects, livestock, wildlife and on the environment (Fatope *et al*., 1993; Funda, 2007). The present investigations have helped us to focus on some bioactive extracts, which possess larvicidal and insecticidal activities. These active extracts could be used for obtaining new leads to isolate bioactive pesticidal molecules from marine resources. Based on the results the most promising extract was ME that showed the best larvicidal, reproductive and biochemical activities. These promising results in relation with insecticidal activity open the way for complementary investigation in order to purify and identify active molecules.

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