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Mosquito larvicidal and ovicidal activity of puffer fish extracts against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae)

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Abstract. The extracts of liver (LE), ovary (OE), skin (SE) and muscle (ME) tissues of four species of puffer fishes *viz*., *Arothron hispidus*, *Lagocephalus inermis*, *Lagocephalus scleratus* and *Chelonodon patoca* were evaluated against larvae and eggs of three mosquito vectors, *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. The LC_{50} values were 1194.26, 1382.73 (LE); 1421.42, 1982.73 (OE); 7116.86, 15038.98 (ME) and 10817.8 ppm (SE) for *An. stephensi* and *Cx. quinquefasciatus* respectively for *A. hispidus*. In the case of *L. inermis*, the LC_{50} values were 1163.83, 1556.1 and 2426.38 (LE); 1653.53, 2734.74 (OE); 6067.47 (ME) and 10283.04 ppm (SE) for *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* respectively. The LC_{50} values were 1509.98, 1608.69 (LE) and 1414.9, 2278.69 ppm (OE) for *An. stephensi* and *Cx. quinquefasciatus* respectively for the extracts of *L. scleratus*. In the case *C. patoca* extracts the LC_{50} values were 1182.29, 1543.00, 2441.03 (LE) and 1076.13, 2582.11 ppm (OE) for *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* respectively. OE and LE of all puffer fishes exhibited zero percent egg hatchability from 600 to 1000 ppm against eggs of *An. stephensi* and *Cx. quinquefasciatus*. This study shows that puffer toxins are effective in killing the larvae and eggs of mosquitoes.

INTRODUCTION

Mosquitoes transmit many tropical and subtropical diseases such as dengue fever, yellow fever, malaria, filariasis and Japanese encephalitis (Service, 2004). *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* 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). Vector control is definitely the best method of protecting the community against the vector borne diseases (Sharma, 2001). Personal protection from biting mosquitoes and other haematophagous arthropods is the first choice of defense against the infectious disease (WHO, 2004). 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.

Botanical insecticides have long been sought as alternatives to synthetic chemical insecticides for insect control because botanicals reputedly pose little threat to the environment or to human health. Recently much importance is given to biocontrol agents viz., microbials (Mittal, 2003; Isman, 2006) such as *Bacillus thuringiensis* var. *israelensis (Bti)* and *Bacillus sphaericus* for mosquito vector control.

Venoms are increasingly being seen as a source of novel biological agents in areas as diverse as medicine and agriculture. Numerous spider venoms have been examined for activity in insects. Their biologically active components are usually polyamines and peptides (Entwistle *et al.*, 1982; Quistad *et al.*, 1990; Skinner *et al.*, 1990; Johnson *et al.*, 1998). In general, polyamine toxins cause faster-acting, reversible incapacitation of the target insect, whereas peptide toxins are associated with longer-term effects. Venoms from scorpions and ants are rich sources of insect-active peptides (Eitan *et al.*, 1990; Zilberberg *et al.*, 1991).

Secondary metabolites of marine organisms differ from that of terrestrial organisms. Bioactive compounds isolated from marine organisms exhibited 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* Hendersen and sea cucumber *Holothuria scabra* Jaeger extracts have been reported (Narsinh *et al.*, 2004).

Puffer fishes are very unique looking fishes. The puffer fish has the remarkable ability to expand its body extremely quickly when faced with danger. Puffer fishes were caught concomitantly with other commercially important fishes by fishermen and used to discard since they cause food poisoning. In the present paper we report the larvicidal and ovicidal potential of the puffer fish extracts (PFEs) against three species of mosquito vectors, *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

MATERIALS AND METHODS

Collection of puffer fishes

Four species of puffer fishes viz., *Arothron hispidus*, *Lagocephalus inermis*, *Lagocephalus scleratus* and *Chelonodon patoca* were collected from different fishing landing areas of Tamil Nadu and Pondicherry, southeast coast of India. All the samples were transported in ice boxes to the laboratory at Vector Control Research Centre and subsequently kept frozen at -20°C until use.

Preparation of the crude extract

The puffer fishes were partially thawed and excised the tissues of muscle, liver, ovary and skin excised and collected separately. Each specimen was minced immediately to minimize the effect of drying and was extracted separately. Tissue grinding was done using mortar and pestle. After grinding, the tissues were soaked overnight in water (1.5 times by weight of the tissues) and weak organic acid of 1% acetic acid (0.5% weight of the tissues) and then filtered by using Whatman no.1 filter paper and collected the lixiviated solution. The same tissues were extracted thrice in the same manner in order to extract as much toxin as possible. The lixiviated solutions were heated (70-95°C) to remove the coagulated soluble proteins (scleroproteins). The pH of the lixiviated solution was adjusted to 7.0 using an aqueous solution of weak base (ammonia). Water was removed from the crude extract by heating under reduced pressure to yield a viscous dark brown residue. On an average, 2.5 to 3.5 gm of crude extract was obtained for each 100 gm of tissues. The crude extract was lyophilized and used for bioassay against vector mosquitoes.

Test organisms

Eggs and larvae of vector mosquitoes *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were obtained from rearing and colonization division of Vector Control Research Centre (VCRC) at Pondicherry, India.

Larvicidal activity

Larvicidal evaluation was carried out at different concentrations by preparing the required stock solutions by following the standard procedure (WHO, 2005). Appropriate serial dilutions were made

from the stock solution and the desired concentrations of the test solution were achieved by adding 1.0 ml of an appropriate stock solution to 99 ml of tap water. The mosquito larvae were exposed to different concentrations of the test solution ranging from 500 to 16000 ppm. Four replicates for each concentration were maintained. Batches of twenty five late third instar larvae were transferred into each disposable cup containing 100 ml of water. Tap water alone was used as control. The bioassays were performed at room temperature (27 ± 1°C). The larval mortality in both treated and control was recorded after 24h exposure period.

$$\text{Mortality (\%)} \frac{X - Y}{X} 100$$

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample. LC_{50} and LC_{90} values were calculated by probit analysis (Finney, 1971) using software SPSS Version 16.

Ovicidal activity

Evaluation of the puffer fish extracts for ovicidal activity was carried out by following the method of Su & Mulla (1998). Eggs were exposed to five different concentrations ranging from 200 to 3000 ppm. The desired concentrations of the test solutions were achieved by adding 1.0 ml of an appropriate stock solution to 99 ml of tap water. Each egg raft containing 100 eggs of *Cx. quinquefasciatus* and hundred eggs of *Ae. aegypti* and *An. stephensi* (14 to 18 hr old) were exposed to each dose of extract for 48 hr. Counting of eggs was done under a microscope. Tap water without test solution served as control. Four replicates for each concentration were maintained. After 48 hr of incubation, the egg rafts or eggs exposed to each concentration were transferred to distilled water cups. The hatch rates were calculated by the following formula.

$$\frac{\text{Number of hatched larvae}}{\text{Total number of eggs}} \text{ X } 100$$

RESULTS

The results of the liver, ovary, skin and muscle extracts of all the four species of puffer fishes evaluated against all the three species of mosquito larvae are given in Table 1. The LC_{50} values were 1194.26, 1382.73 (liver extract-LE); 1421.42, 1982.73 (ovary extract-OE); 7116.86, 15038.98 (muscle extract-ME) and 10817.8 ppm (skin extract-SE) for *An. stephensi* and *Cx. quinquefasciatus* respectively for the extracts of *A. hispidus*. In the case of *L. inermis*, the LC_{50} values were 1163.83, 1556.1 and 2426.38 (LE); 1653.53, 2734.74 (OE); 6067.47 (ME) and 10283.04 ppm (SE) for *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* respectively. The LC_{50} values were 1509.98, 1608.69 (LE) and 1414.9, 2278.69 ppm (OE) for *An. stephensi* and *Cx. quinquefasciatus* respectively for the extracts of *L. scleratus*. In the case *C. patoca* extracts the LC_{50} values were 1182.29, 1543.00, 2441.03 (LE) and 1076.13, 2582.11 ppm (OE) for *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* respectively.

The LC_{50} values for liver, ovary, skin and muscle tissue extracts of *A. hispidus*, *L. inermis*, *L. scleratus* and *C. patoca* against the larvae of three mosquito vectors *viz.*, *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* ranged from 1076 to 15039ppm. In the case of A. *hispidus* and *L. inermis* the liver extract was more effective than ovary extract followed by muscle extract and skin extract against *An. stephensi* and *Cx. quinquefasciatus* larvae respectively. The ovary extract of *L. scleratus* and *C. patoca* were more effective than liver extract against *An. stephensi* larvae.

Results of ovicidal evaluation (Table 2) showed that when the eggs were treated with puffer fish extracts hatchability of the eggs was affected. The eggs of *An. stephensi* were completely inhibited from hatching when the eggs were treated with 600ppm of OE and 800ppm of LE of *A. hispidus*. The OE and LE of *L. inermis* exhibited complete inhibition of egg hatchability at 600ppm and 1000ppm against the eggs of *An. stephensi*. The OE of *L. scleratus* exhibited complete inhibition of egg hatching at 800ppm against *An.*

stephensi. The OE and LE of *C. patoca* exhibited complete inhibition of egg hatchability at 800ppm against the eggs of *An. stephensi* and at 1000ppm against *Cx. quiquefasciatus*. In the case of *Ae. aegypti* up to 1000ppm no ovicidal effect was observed.

DISCUSSION

Chemical insecticides have been used arbitrarily during the past few decades for crop protection and vector borne disease control which has led to the development of resistance in many insect species including mosquito vectors (Zlotkin, 1999; Hemingway & Ranson, 2000; Soderlund & Knipple, 2003). This warrants the need for the development of alternate tools/strategies for mosquito control.

Majority of the mosquito control programmes 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.

A toxin has been reported to be present in the liver and ovaries of puffer fishes which

Table 1. Larvicidal activity of puffer fish extracts against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*

Puffer fish species	Extract	Species	LC_{50} (ppm)	LC_{90} (ppm)	95% Confidence Limits UCL-LCL (ppm)	Slope	χ^2
A. hispidus	Liver	*An. stephensi*	1194.26	2232.74	947.78-1507.15	99.01	0.444
		Cx. quinquefasciatus	1382.73	2181.47	1205.13-1557.15	91.42	0.001
	Ovary	*An. stephensi*	1421.42	2574.57	1148.86-1655.12	118.91	1.894
		Cx. quinquefasciatus	1982.73	3233.24	1732.69-2252.89	132.38	0.476
	Muscle	*An. stephensi*	7116.86	13367.50	5757.89-8451.30	644.76	1.817
		Cx. quinquefasciatus	15038.98	20722.00	13564.5-17479.85	983.02	0.428
	Skin	*An. stephensi*	10817.80	20360.60	9012.12-13170.57	877.07	0.0003
		An. stephensi	1163.83	1954.46	945.89-1351.24	104.89	1.143
L. inermis	Liver	*Cx. quinquefasciatus*	1556.10	2563.16	1326.69-1769.22	113.32	1.989
		Ae. aegypti	2426.38	3668.61	2171.07-2774.06	146.64	0.185
	Ovary	*An. stephensi*	1653.53	3141.79	1301.47-1947.15	136.80	2.817
		Cx. quinquefasciatus	2734.74	4561.69	2383.48-3235.84	187.16	0.079
	Muscle	*An. stephensi*	6067.47	11949	4705.54-7333.12	642.16	0.722
	Skin	*An. stephensi*	10283.04	21015.9	8290.39-12846.46	885.263	1.851
L. scleratus	Liver	*An. stephensi*	1509.98	2702.14	1240.51-1749.43	115.26	2.699
		Cx. quinquefasciatus	1608.69	2543.72	1395.65-1812.88	111.08	1.826
	Ovary	*An. stephensi*	1414.90	2522.70	1151.37-1642.11	118.84	1.549
		Cx. quinquefasciatus	2278.69	3628.95	2013.96-2619.23	110.85	0.664
C. patoca	Liver	*An. stephensi*	1182.29	2006.48	959.42-1374.07	104.95	1.615
		Cx. quinquefasciatus	1543.00	2686.92	1286.50-1775.55	119.06	3.102
		Ae. aegypti	2441.03	3753.34	2173.61-2816.91	145.41	0.649
	Ovary	*An. stephensi*	1076.13	2220.38	731.81-1321.37	114.58	1.025
		Cx. quinquefasciatus	2582.11	4121.47	2265.03-3098.23	170.59	1.576

Table 2. Ovicidal activity of puffer fish extracts against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*

Puffer fish species	Extract	Mosquito Species	Percentage of egg hatchability					
			Concentration (ppm)					
			Control $\overline{X}$ ± SD	200 $\overline{X}$ ± SD	400 $\overline{X}$ ± SD	600 $\overline{X}$ ± SD	800 $\overline{X}$ ± SD	1000 $\overline{X}$ ± SD
A. hispidus	Liver	*An. stephensi*	98.7±0.5	80.0±2.5	49.0±2.1	16.5±1.2	0	0
		Cx. quinquefasciatus	100±0.0	87.0±2.4	65.7±4.3	32.2±3.5	17.2±2.6	0
		Ae. aegypti	99.2±0.5	98.5±0.5	99.2±0.5	99.5±0.5	94.7±2.2	78.2±1.7
	Ovary	*An. stephensi*	98.7±0.9	70.5±3.8	15.2±1.2	0	0	0
		Cx. quinquefasciatus	98.5±0.5	91.7±2.6	70.0±3.9	25.2±3.4	5.5±1.2	0
		Ae. aegypti	100±0.0	99.2±0.9	99.5±0.5	99.2±0.9	96.2±1.2	77.2±5.1
L. inermis	Liver	*An. stephensi*	100±0.0	57.5±2.3	33.5±1.7	25.2±1.8	10.5±1.2	0
		Cx. quinquefasciatus	100±0.0	86±4.3	46.7±2.3	27.2±2.2	12.5±2.3	0
		Ae. aegypti	100±0.0	100±0.0	99.2±0.9	86.5±2.6	68.5±5.5	47.5±6.7
	Ovary	*An. stephensi*	98±1.4	70.5±6.3	16±1.4	0	0	0
		Cx. quinquefasciatus	100±0.0	91.5±2.6	68.5±6.0	39.7±1.7	5.5±1.2	0
		Ae. aegypti	100±0.0	99.7±0.5	99.5±0.5	95.2±2.2	79±1.8	75.5±7.7
L. scleratus	Liver	*An. stephensi*	98.5±1.2	80±1.8	38.2±2.2	12.5±2.2	2.7±1.7	0
		Cx. quinquefasciatus	99.7±0.5	99.5±1.0	80.2±0.9	51.5±2.6	20.5±1.2	11.5±1.1
		Ae. aegypti	100±0	99.7±0.5	79.5±1.2	60.7±2.0	47.7±2.5	36.7±1.71
	Ovary	*An. stephensi*	97.5±1.2	58.7±1.7	31.5±2.5	6.5±3.1	0	0
		Cx. quinquefasciatus	99.7±0.5	97.5±1.2	82±2.1	58.5±2.1	29.7±2.5	13.5±2.0
		Ae. aegypti	100±0	98.5±0.5	99±0.8	90±0.82	70.5±1.2	40.2±4.9
C. patoca	Liver	*An. stephensi*	98.5±1.2	79.5±1.2	53.2±2.7	27.2±2.2	0	0
		Cx. quinquefasciatus	99.0±0.8	93.0±2.1	73.7±3.4	57.2±2.2	21.5±1.2	0
		Ae. aegypti	100±0	89.7±1.7	62.5±2.08	50.5±2.0	28.5±2.0	17.5±1.2
	Ovary	*An. stephensi*	97.75±1.5	28.5±3.1	15.7±3.4	1.5±1.0	0	0
		Cx. quinquefasciatus	100±0.0	99.5±0.5	89.0±1.4	68.0±2.1	28.5±2.0	0
		Ae. aegypti	99.5±0.5	97.75±1.5	94.7±1.2	90.5±0.5	79.5±1.2	61.2±1.8

has effect on ion channels (Fuchi *et al*., 1991; Noguchi & Arakawa, 2008). Tetradotoxin is a heat stable, water soluble and non-protein quinazoline derivative (Sorokin, 1973). Tetrodotoxin acts on the central and the peripheral nervous systems. In spite of the toxic nature of the puffer fish and its recognized ill effects, it is a delicacy in Japan that is prepared by licensed puffer fish cooks (Lange, 1990). The toxin tetrodotoxin 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 anaesthesia 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 eggs. A work has been initiated to exploit the possibility of using puffer fish extracts for mosquito larval control.

In the present study, different parts *viz*., liver, ovary, skin and muscle were separated from these fishes and extracted with 1% acetic acid, cleared the scleroproteins and removed the solvent under reduced pressure followed by neutralization and lyophilization. The lyophilized material was screened against eggs and larvae of three mosquito species viz., *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* as per standard procedure (WHO, 2005). The results showed that the liver and ovary extracts from all the four fishes were more effective in killing the mosquito larvae than the skin and muscle extracts.

Among the extracts from four species of puffer fishes, the ovary extract of *C. patoca* was found to be the effective extract as larvicide. The ovary extracts *A. hispidus* and *L. inermis* were effective as ovicide compared to other extracts. Among the three mosquito species *An. stephensi* was most

susceptible (both larvae and eggs) species. Earlier reports show that in most marine puffers, high concentrations of tetrodotoxin are found in livers and ovaries/eggs, but significant amounts are also detected in digestive tissue, muscles and skin (Fuchi *et al.*, 1991; Noguchi & Arakawa, 2008). The results of this study also show that the ovary extracts and liver extracts are more effective than the muscle and skin extracts indicating that the toxin distribution varies in different tissues.

Earlier reports show that animal toxins are also good sources of insecticidal agents as in the case of phytochemicals and microbial metabolites. The characterization of an insect-active peptide toxin from the venom of the spider *Phoneutria nigriventer* was reported (Figueiredo *et al.*, 1995). Six insecticidal peptides were isolated from the venom of *Aptostichus schlingeri* (Skinner *et al.*, 1990). The sponge extracts of *Psammaplysilla purpurea* and *Haliclona cribricutis* was found most effective against *A. aegypti* larvae (Venkateswara Rao *et al.*, 2008). Earlier studies reported (Samidurai *et al.*, 2011) the toxicity of nereistoxin from *Lumbriconereis heteropoda* to the mosquito vectors *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*.

Recently much importance is given to microbial based biocontrol agents such as *B. thuringiensis* var. *israelensis (Bti)* and *B. sphaericus* (Mittal, 2003). Laboratory tests of aqueous formulation of *B. thuringiensis* var. *israelensis* showed that the aqueous suspension was effective against *Cx. quinquefasciatus* than *Ae. aegypti* and *An. stephensi*, the respective LC_{50} values being 0.046, 0.060 and 0.190 ppm (Dominic Amalraj *et al.*, 2000). Tiwari *et al.* (2011) evaluated a water-dispersible granular formulation biolarvicide *B. thuringiensis* var. *israelensis* (Bti, H-14 serotype; VectoBac® WDG) in the laboratory and also in the field against *Anopheles culicifacies* and *An. stephensi*. The LC_{50} values against *An. culicifacies* and *An. stephensi* were 0.348 and 1.008ppm respectively. However, high level of *B. sphaericus* resistance to mosquito larvae was reported (Rao *et al.*, 1995; Yuan *et al.*, 2000) and no more recommended for mosquito control operations.

Botanical insecticides have long been sought as alternatives to synthetic chemical insecticides for insect control because botanicals reputedly pose little threat to the environment or to human health (Isman, 2006). Though the mosquito larvicidal activity of a number of plant extracts have been reported (Carvalho *et al.*, 2003; Cavalcanti *et al.*, 2004; Shaalan *et al.*, 2005; Isman, 2006; Nisha *et al.*, 2009; Samidurai *et al.*, 2009; Govindarajan *et al.*, 2011) few like Neem and Pyrethrum only commercially exploited. The Neem (*Azadirachta indica*) (Schmutterer, 1995) and Pyrethrum (Casida, 1973) extracts and the derived products from them have shown a variety of insecticidal properties on a broad range of insect species.

Previous literature indicates 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. In the case of sponge extracts *P. purpurea* and *Haliclona cribricutis* exhibited mosquito larvicidal activity at <50ppm against *Ae. aegypti* (Venkateswara Rao *et al.*, 2008) while sea cucumber and prawn extracts were effective at higher concentrations (>1000ppm). The current study also shows the mosquito larvicidal and ovicidal activity of puffer fish extracts that are comparable to that of prawn and sea cucumber extracts (Narsinh *et al.*, 2004). This study shows that puffer toxins are effective in killing the larvae and eggs of mosquitoes. However, its use may be limited to polluted water mosquito control (*Cx. quinquefasciatus)* owing to the possible risk of poisoning to humans and animals due to tetrodotoxin in fresh water usage.

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