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A Laboratory based study on the Larvicidal effects of Aquatain, a Monomolecular Film and Mousticide™ [Trypsin Modulating Oostatic Factor [TMOF-*Bti*] formulation for the control of *Aedes albopictus* (Skuse) and *Culex quinquefasciatus* Say in Pakistan

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Abstract
Experiments were performed to test the efficacy of Mousticide™, a combination of two natural substances Trypsin-modulating oostatic factor [TOMF] *Bacillus thuringiensis israelensis* [*Bti*] and Aquatain, against larval late third early four of *Aedes albopictus* and *Culex quinquefasciatus* under laboratory conditions. The results of the study have established the effectiveness of the products tested. The aquatain has maximum mortality trend of *Aedes albopictus* and *Culex quinquefasciatus* by different treatments (0.017 ml, 0.035 ml, 0.070 ml and 0.14 ml) at their maximum concentration (0.14 ml) after 1 hour was (20% and 17% respectively) and after 12 hours (100%). The TMOF-Bti has maximum mortality trend of *Aedes albopictus* and *Culex quinquefasciatus* by different treatments (10 mg, 25 mg, 40 mg and 100 mg) at their maximum concentration (100 mg) after 1 hour was (10% and 12% respectively) and after 24 hours (100%). All the concentrations of Aquatain and TMOF-Bti caused high mortality (95-96%) within 12 hour of treatment and 100% mortality until 24 hours after treatment. Our results have further revealed that Aquatain is more effective as compared to TMOF-*Bti* in controlling larval stages of test mosquitoes.

Keywords: *Aedes albopictus*, *Culex quinquefasciatus*, Mousticide, Aquatain and Trypsin-modulating oostatic factor, *Bacillus thuringiensis israelensis* (*Bti*).

1. Introduction
Mosquitoes have fundamental importance among insect pests as being medically important vectors of dreadful diseases to humans and animals. These vectors have the potential to feed on more than one individual, during a single gonotropic cycle [1, 2, 3]. More than 500 million people in the world grieve from these tropical diseases. It has been reported that one to two million deaths occurs annually due to malaria, the world's most important and dreadful tropical disease [4]. Globally, half of the population is at risk due to dengue infection. Annually, 50 million dengue infections are recorded and about 2 – 5 billion people live in dengue endemic countries [5]. Unlikely most victims are seen among children, pregnant women and other persons with potential to fight off the disease [6].
Culex quinquefasciatus is common, widespread and an urban mosquito with endophilic and anthropophilic behaviour [7]. It transmits St. Louis encephalitis virus, West Nile virus, Rift Valley fever virus and bird malaria and is known to invade new regions as a result of ship transportation [8]. *Culex* sp. despite its diseases causing behaviour is not known in Pakistan for the same besides its vast prevalence in dirty and polluted waters as compared to *Anopheles* sp. and *Aedes sp*. From last 13 years of research indicate that 3.7 million larvae of *Culex sp.* mosquitoes collected from polluted habitats [9]. However, *Aedes albopictus* and *Aedes aegypti* are vectors of more than 30 viruses [10] which breeds in many artificial containers, bamboo, stumps [11], discarded tires [12], tree holes [13] and vases.

For public health concern, the dengue fever is becoming more vital in tropical and sub-tropical parts of the world [14]. Since 2005, Bangladesh, India and Pakistan have been suffering outbreak of dengue fever. It became an endemic in Southeast Asia [15]. Pakistan is at high risk of being hit by large epidemics because of overcrowded cities, hazardous drinking water, open drainage system, poor sanitation conditions and large number of refugees [16].

Since 2005, the dengue fever is becoming a major threatening issue in Pakistan. An outbreak of DHF (Dengue Hemorrhagic Fever) was first reported in Karachi in 1994 [17]. In 2003, its outbreak was detected in sub-mountainous areas [9]. From three districts of Pakistan, only 699 dengue cases were reported from 1995 to 2004 [18]. A sudden rise in cases of dengue fever was witnessed in year 2005 in Karachi. During the year 2010, 16,580 cases of dengue fever and 257 deaths were reported in Lahore and about 5000 survivors of dengue fever whereas 60 deaths were reported from rest of the areas of Pakistan [19]. In 2011, 21292 number of dengue fever confirmed cases and 352 deaths were reported from Punjab. In following year 2012, 258 dengue confirmed cases and 01 death were reported. In 2013, 18 deaths were reported from 2661 dengue confirmed cases. In 2014, only 01 death were reported out of 1440 confirmed cases from Punjab. In 2015, 08 deaths were reported out of 4348 dengue fever confirmed cases. In 2016, 5059 number of confirmed cases of dengue fever and 03 deaths were reported from the Punjab province of Pakistan [20].

Chemicals remain as the most powerful tools in combating the mosquito population. Despite the dependency on these chemical the choice is limited. It is obvious in certain parts of the world that resistance to organophosphates, carbamates, chlorinated hydrocarbon insecticides and pyrethroids etc. [21] is becoming an alarming factor.

Aedes aegypti (L.), the primary vector of dengue fever, has developed resistance to various insecticides [21]. Therefore, it is necessary, from time to time, to monitor the susceptibility status of mosquito vectors to the insecticides used in the control measures besides finding alternate options. The use of the eco-friendly compounds for mosquito larval can thus help to reduce the burden on the use of pesticides.

TMOF is an insect hormone originally isolated from the ovaries of *Aedes aegypti* (Diptera: Culicides), which regulates trypsin biosynthesis in the mosquito digestive system [22]. TMOF has been shown to inhibit the growth and development of mosquito larvae feeding on this peptide, leading to death from hunger [23]. Whereas Aquatain, which is the monomolecular layer differs from each other in the mosquito life cycle. All stages that occur in contact with the water surface (e.g., eggs, larvae, pupae, emerging adults, and ovipositing females) are affected by the lowered surface [24, 25] and thus control adult and larvae [26, 27, 28, 29, 30].

Therefore present study has been undertaken to test the efficacy of Mousticide™ (Trypsin-modulating oostatic factor (TMOF-*Bti*) and Aquatain against third and fourth instars larvae of mosquitoes as these two eco-friendly compounds have never been used in Pakistan to control the mosquitoes.

Materials and Methods

The efficacy of different products such as Mousticide™ [Trypsin-modulating oostatic factor [TMOF-*Bti*] and Aquatain were tested against late third and earlier fourth instar laboratory strain larvae of *Aedes albopictus* and *Culex quinquefasciatus* being vectors of human and animal diseases.

Efficacy Testing Procedure

Bioassay test against *Aedes albopictus* and *Culex quinquefasciatus* were carried out under laboratory condition at a temperature of 28 ± 2 ºC, 65% RH and a photoperiod of 14L: 10D hours.

Aquatain and TOMF-*Bti* were used at different concentrations of 0.017 ml, 0.035 ml, 0.070 ml and 0.14 ml and 10 mg, 25 mg, 40 mg and 100 mg, respectively, against 3rd and 4th instar larvae of mosquito (*Aedes albopictus* and *Culex quinquefasciatus*). Bioassay test was carried out on 20 larvae which were placed in 250 ml beaker containing test solution. Percent mortality was recorded after 1, 3, 6, 12 and 24 hours.

The data collected was subjected to statistical analysis by Probit Analysis [31]. For larval bioassay under laboratory conditions, the differences between the LC_{50} and LC_{90} values are considered significant if their fiducial limits (95%) did not overlap [32]. Probit Analysis at 95% after different time intervals was calculated by using SPSS 19.

Results

The results of different Aquatain, a Monomolecular Film and Trypsin Modulating Oostatic Factor [TMOF-Bti] chemicals were recorded in terms of mortality against third and fourth instar larvae of *Aedes albopictus* (Skuse) and *Culex quinquefasciatus* Say under laboratory conditions.

Aquatain: Aquatain gave maximum mortality trend against *Aedes albopictus* and *Culex quinquefasciatus* after 12 hours in different treatments at their maximum concentration (0.14 ml). For *Aedes albopictus* after 1 hour it was (20%), after 3 hours (42%), after 6 hours (85%) and after 12 hours (100%) and for *Cxlex quinquefasciatus* after 1 hour it was (17%), after 3 hours (37%), after 6 hours (77%), after 12 hours (100%) (Figure 1).

LC_{50} and LC_{90} values of Aquatain at different time interval against third/fourth instar larvae of *Aedes albopictus* were recorded and subjected to Probit analysis (Table 1). This indicated that after 12 hours the desired results were achieved that yielded 50% and 90% mortality of the third/fourth instar larvae of *Aedes albopictus* (Figure 1).

LC_{50} and LC_{90} values of Aquatain at different time interval against third/fourth instar larvae of *Culex quinquefasciatus* were recorded and subjected to Probit analysis (Table 1). This indicated that after 12 hours the desired results were achieved that yielded 50% and 90% mortality of third/fourth instar larvae of *Culex quinquefasciatus* (Figure 1).

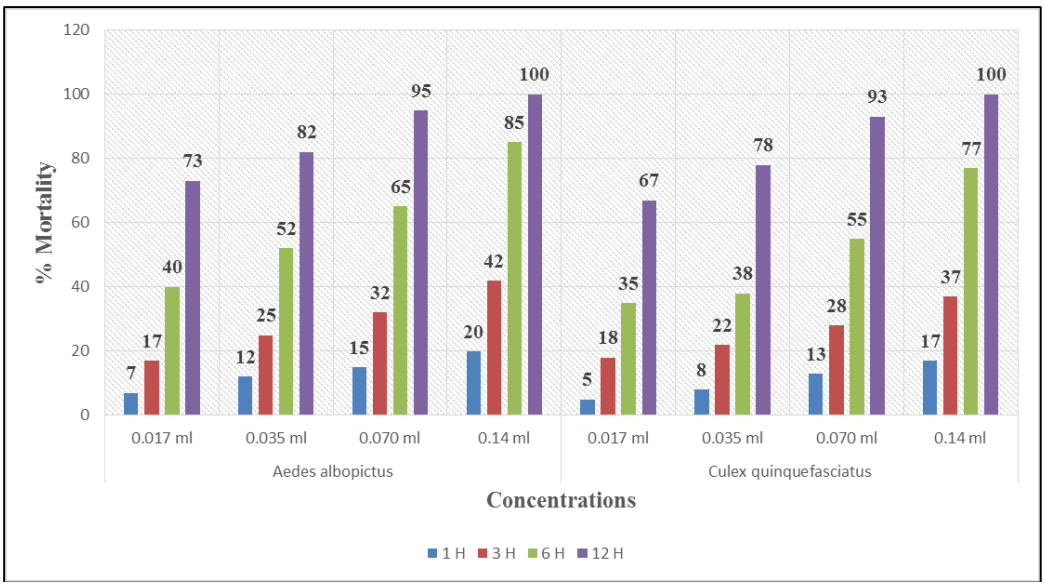

Fig 1: % Mortality of Aquatain at different concentration against third and fourth instar larvae of *Aedes albopictus* and *Culex quinquefasciatus*.

Table 1: LC_{50} and LC_{90} value of Aquatain against third and fourth instar larvae of *Aedes albopictus* and *Culex quinquefasciatus*.

	Time (Hours)	LC_{50}	Slope ± S.E	χ^2	P value	LC_{90}	Slope ± S.E	χ^2	P value
Aedes albopictus	1	2199.47	0.692 ± 0.315	0.106	0.948	156032.21	0.692 ± 0.315	0.106	0.948
	3	255.11	0.871 ± 0.262	0.049	0.976	9436.20	0.871 ± 0.262	0.049	0.976
	6	29.43	1.35 ± 0.262	1.038	0.595	260.78	1.35 ± 0.262	1.038	0.595
	12	9.39	1.91 ± 0.418	2.21	0.331	43.86	1.91 ± 0.418	2.21	0.331
Culex quinquefasciatus	1	2509.30	0.553 ± 0.347	0.081	0.960	130061.03	0.553 ± 0.347	0.081	0.960
	3	519.83	0.637 ± 0.263	0.107	0.948	53568.91	0.637 ± 0.263	0.107	0.948
	6	46.25	1.23 ± 0.253	2.39	0.303	502.67	1.23 ± 0.253	2.39	0.303
	12	12.03	2.04 ± 0.396	2.15	0.340	50.97	2.04 ± 0.396	2.15	0.340

Mousticide™ [TMOF-*Bti*]: TMOF-*Bti* yielded different mortality trends against *Aedes albopictus* and *Culex quinquefasciatus* in different treatments after 24 hours. For *Aedes albopictus* mortality after 1, 3, 6, 12 and 24 hours was 10%, 42%, 57%, 93% and100%. Similarly for *Culex quinquefasciatus* mortality after 1, 3, 6, 12 and 24 hours was 12%, 38%, 57%, 89% and 100% (Figure 2).

LC_{50} and LC_{90} values of TMOF-*Bti* at different time interval against third/fourth instar larvae of *Aedes albopictus* were recorded and subjected to Probit analysis (Table # 2). This indicated that after 24 hours the desired result were achieved that yielded 50% and 90% mortality of the third/fourth instar larvae of *Aedes albopictus* (Figure 2).

LC_{50} and LC_{90} values of TMOF-*Bti* at different time interval against third/fourth instar larvae of *Culex quinquefasciatus* were recorded and subjected to Probit analysis (Table # 2). This indicated that after 24 hours the desired results were achieved that yielded 50% and 90% mortality of the third/fourth instar larvae of *Culex quinquefasciatus* (Figure # 02).

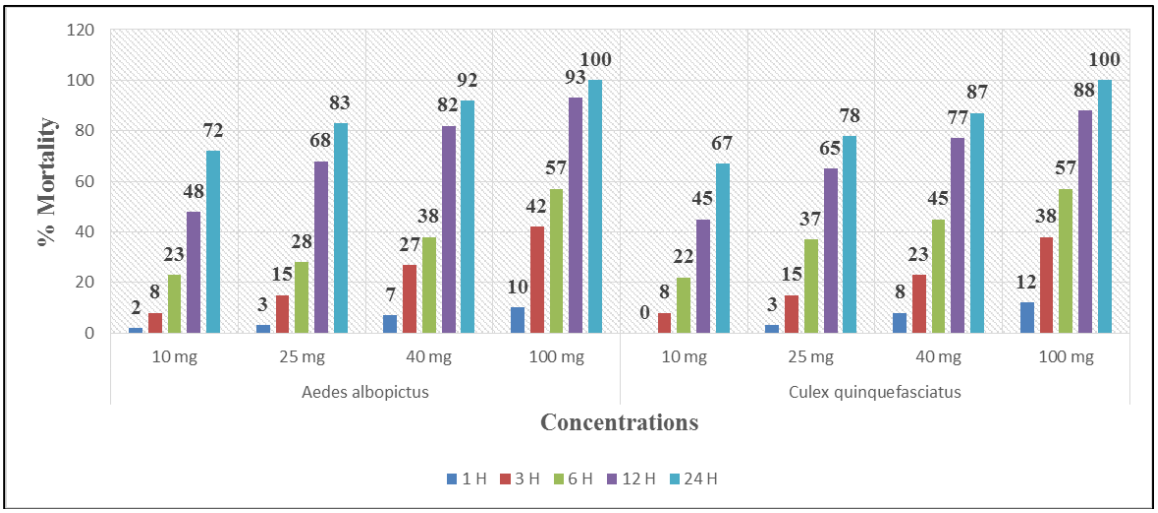

Fig 2: % Mortality of TMOF-*B.t.i* at different concentration against third and fourth instar larvae of *Aedes albopictus* and *Culex quinquefasciatus.*

Table 2: LC_{50} and LC_{90} value of TMOF-*Bti* against third and fourth instar larvae of *Aedes albopictus* and *Culex quinquefasciatus*.

	Time (Hours)	LC_{50}	Slope ± S.E	χ^2	P value	LC_{90}	Slope ± S.E	χ^2	P value
Aedes albopictus	1	2955.52	0.853 ± 0.407	0.204	0.903	94128.41	0.853 ± 0.407	0.204	0.903
	3	146.90	1.21 ± 0.272	0.421	0.810	1654.42	1.21 ± 0.272	0.421	0.810
	6	76.97	0.929 ± 0.238	0.954	0.621	1843.85	0.929 ± 0.238	0.954	0.621
	12	11.08	1.55 ± 0.275	0.254	0.881	74.33	1.55 ± 0.275	0.254	0.881
	24	5.08	1.63 ± 0.361	2.22	0.328	30.84	1.63 ± 0.361	2.22	0.328
Culex quinquefasciatus	1	747.15	1.25 ± 0.448	1.67	0.434	7855.15	1.25 ± 0.448	1.67	0.434
	3	186.79	1.11 ± 0.273	0.133	0.936	2617.86	1.11 ± 0.273	0.133	0.936
	6	60.72	0.948 ± 0.237	0.207	0.902	1363.47	0.948 ± 0.237	0.207	0.902
	12	12.37	1.34 ± 0.257	0.098	0.952	112.01	1.34 ± 0.257	0.098	0.952
	24	6.36	1.58 ± 0.328	3.71	0.156	40.79	1.58 ± 0.328	3.71	0.156

Discussion

The results of the study have established the effectiveness of these products. There are also significant differences among them at different application doses and time in terms of mortality percentage of both mosquito species. The mortality ratio of mosquito species increased in association with time and of compound concentrations. There was also significant interaction among times and of insecticidal compounds used. The aquatain has maximum larval mortality trend of *Aedes albopictus and Culex quinquefasciatus* in different treatments at their maximum concentration (0.14 ml) after 12 hours (100%). The TMOF-*Bti* has maximum mortality trend in larval stages of *Aedes albopictus and Culex quinquefasciatus* by different treatments at their maximum concentration (100 mg) after 24 hours (100%).

The present study has indicated that MMF (monomolecular film) was able to control multi stages of *Aedes albopictus* (L.) and *Culex quinquefasciatus* which is in agreement with previous studies [33, 34]. The effects of MMF are not only limited to *Aedes* and *Culex*, but also *Anopheles* mosquitoes were reported to have increased mortality in laboratory and field studies [35, 36].

The observations on larval behaviour changes revealed increased nibbling of their tails, decreased feeding, leading to the accumulation of food in the tray. Low food consumption in larvae resulted in both smaller-sized larvae and prolonged stage transformation compared to the control group. Similar study indicate that larvae and pupae exposed to MMF emerged to be smaller adults with lower egg-laying capacity, suggesting that MMF probably reduced their vectorial capacity. The application of MMF on the water surface killed the aquatic stages and affected oviposition of the gravid female mosquitoes [37, 38]. A similar study showed that gravid female mosquitoes avoided ovipositing on an MMF-coated water surface. The MMF effect on the water surface was unlikely to have an impact on female *Aedes* because they lay eggs on the inner wall of the oviposition cup above the water-line [33, 39]. However, another similar study indicate that a group of female mosquitoes introduced in each test cage could increase the risk of detecting pseudo preferences, especially if group sizes were small. Thus, further investigation of the oviposition preference experiments of MMF should involve a single mosquito per cage with sufficient replication [40]. A similar study evaluated that the efficacy of the larvicidal and pupicidal agent (Agnique®) - MMF was evaluated against larvae of *An. arabiensis* and *Culex* (Diptera: Culicidae) under field conditions. They concluded that Agnique® can perform very effectively against L3-L4 instars and pupae of *An. arabiensis* for only 1 week, and 3 to 4 days against L1-L2 instars of *Culex* spp [41]. The similar studied describe that the asphyxiating effect on larvae and pupae by using aquatain as monomolecular films. It forms flexible layer on a water surface and it can spread on entire water surface. It showed oviposition and larvicidal and pupicidal effects against *An. gambiae* and *Anopheles stephensi* which are the vectors of malaria. Due to water surface stiffness, the female fails to oviposit. This method is cost-effective, safe and resistance-proof tool for vector control [33]. A similar study for effectively interrupting the dengue transmission cycle, as a larvicides and to kill the pupal stages Aquatain AMF should be included in an emergency dengue control program in addition to *Bti*, pyriproxyfen, or temephos [42]. The various formulations of TMOF viz recombinant TMOF yeast cell paste form, TMOF yeast cell dried powder form, a combination of TMOF and *Bti* rice husk, TMOF and *Bti* wettable powder and TMOF and *Bti* mosquito fudge cubes formulated against *Aedes aegypti* larvae in the laboratory. These products had a prolonged residual effect for four weeks of observation. The TMOF and dried yeast powder (Pichia 11 and 12) caused 100% larval mortality after 96 hours of treatment. The TMOF and *Bti* in rice husk, after 1 hour's exposure at different concentration 50 mg, 100 mg and 200 mg shows 85.9%, 87.5% and 94.3% mortality respectively but after 24 hours 100% mortality were observed in all concentration. It showed 100% mortality for 120 hours exposure [43]. The residual effectiveness of (TMOF-*Bti*) formulations against *Anopheles sinensis*, Mousticide™ in Rice Husk, 15% mortality were observed after 1 hrs but it increased to 100% mortality after 24 hours. However, Mousticide™ in wettable powder, after 24 hours of application, the larval mortality recorded were 96.7% which increased to 100% after 72 hours. While Abate™, a neurotoxic chemical only showed 3.33% larval mortality after 24 hours of exposure [44]. The residual effectiveness of trypsin modulating factor-*Bacillus thuringiensis israeliensis* (TMOF-*Bti*) formulations against *Aedes aegypti*, 100% mortality was observed after two weeks in all the concentration [45]. Our result showed that the 100% mortality were observed after 24 hours.

Conclusion

Our study concluded that all concentrations of TMOF-*Bti* and Aquatain caused high mortality (95-96%) within 12 hour of treatment and caused complete mortality (100%) until 24 hours after treatment. From this data, we can conclude that Aquatain is more effective as compare to [TMOF-Bti]. They can be used for the control of mosquitoes and both environmentally friendly.

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