Synergism between Horse Chestnut (*Aesculus Hippocastanum*) Plant and Two Bacterial Larvicide *(Bacillus thuringiensis Serotype* H-14 And *Bacillus sphaericus* in Controlling the Danque Fever Vector*, Aedes aegypti*

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Abstract: The present study deals with the evaluation of efficiency of two bacterial mosquito larvicides and a plant extract one against *Aedes aegypti* larvae when used in combinations with each other under laboratory conditions, is an integrated method of control . The larvicides formulations used were , *Bacillus thuringiensis* H-14 , *B. sphaericus* 1543-4 and a plant water extract of *Aesculus hippocastanum* date collected after 24 hrs of application under laboratory conditions (27±3˚c) comparing the effect of adding the *B. sph*. Or the horse chestnut to larvae pre-treated for 24 hrs with *B.th*. indicated plant extract addition cause significant increase in larval mortality at the first 24 hrs but with increasing the exposure time no increasing in this mortality percentage. Adding *B. th* . H. 14 to larvae pre-treated with *B. sph*. for 24 hrs is better than using plant extract . No significant difference was found between the efficacy of the extract when it added to larvae pre-treated for 24 hrs with *B.th*. or *B* . *sph*.
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1. Introduction

During the past decade, two bacterial mosquito pathogens were used as larvicides in mosquito control programmes, namely, *Bacillus thuringiensis* serotype H-14 and *Bacillus sphaericus* 1543-4. In spite of their relatively high larvicidal activity, yet , the application of the developed commercial formulations under field conditions are still needs further investigations particularly , when these biological control agents are used in integration with other conventional botanical control measures , (Mohan *et al.*, 2001 , Shelton *et al.* , 2002 , Hura *et al.*, 2003 , zhang *et al.* Brar *et al.*, 2006 , Wang *et al.*, 2007. The spore forming gram positive bacterium *B.t.* is an environmentally safe biological insecticide (Rosan *et al.*, 2009). During sporulation *B.T.*produces one or more crystal proteins, which used as biological control agents, Beside using bacteria , botanicals used as safe method also , these botanicals are widely used premium (horse chestnut, *Aesculus hippocastanum* used as an alternative method alone or with bacteria to control the denque fever vector *Aedes aegypti* to evaluate another method (Kumar *et al.*, 2008, Soberon *et al.* , 2009 , Yulin *et al.*, 2010). This plant grow throughout the northern hemisphere although horse chestnut is sometimes called buckeye, it should not be confused with the Ohio or California buckeye trees, which are related but not the same species. For centuries, horse chestnut seeds, leaves, bark and flowers were used in medical requirements.

Horse chestnut seed extract standardized to contain 16 to 20 percent aescin (escin), the active ingredient (national center for complementary and alternative medicine) the nuts contain high concentration of a saponin – class toxins called Aesulin, which is toxic to many animals. In California *Aesculus* kill honey bees. The present work deals with investigating the effect of using mixtures of more than bacterial species as well as mixtures of botanical larvicides and these microbial mosquito larvicides in order to demonstrate the best integrated system that could be used in mosquito control. The aim of the present study is screening of susceptibility of mosquito larvae to botanical and biological (bacterial) between bacterial and botanical mosquito larvicides.

2. Materials and Methods:

1-Test insect

a- ***Aedes aegyptii***

A self perpetuating laboratory colony was raised from a field sampled strain, collected from Jeddah city – Saudi Arabia . The reared colony was maintain under normal day light photoperiods, 27±3˚c and 75±5% RH).Gravid females were allowed to oviposit in plastic cups (10×10cm.) half filled with tap water, the laid eggs were collected daily and separated into bigger enamel pan for larvae hatching. About (25-300) hatched larvae were transferred to round white enamel bowl (35 cm in diameter and 10 cm in height) half filled with tap water . A mixture of bread, dried yeast and dried milk in 2:1:1 ratio, respectively , were ground , sieved and used as larvae food . Pupae were

collected daily by means of a wire mesh and transferred to plastic containers (10×10 cm) half filled with tap water and then introduce inside adult breeding cages . Adult mosquitoes were reared in wooden cages (50×50×50 cm).

Females were fed on blood meals through the introduction of a pigeon into the adult cages. During feeding , cages were covered with black cloth to achieve a more rapid feeding. Plastic cups half filled with tap water were placed inside the cages for egg oviposition a sponge soaked with 10% sugar solution was used for feeding males, from time to time the sponge was replaced by a new one to avoid fungal contamination.

2- Bacterial larvicides

Two mosquito – pathogenic bacteria were used during the present investigation, *Bacillus thuringiensis* serotype H-14 , *Bacillus sphaericus* strain 1593-4 , both bacterial species are belonging to family , Bacillaceae . a commercial formulation in the form of a flowable concentrate under the name of " TEKNAR" , SAN -402 based on *B.th*. H -14 , produced by Sandoz Co. and a *B. sph*. strain 1593-4 in the form of a wetable power produced by SOLVAY- Belgium . Botanical insecticide used was the horse chestnut (premium) , *Aesculus hippocastanum* , prepared by grinding its parts .

3- Bioassays

a)Preparation of the bacterial concentrations :-

For *B.th*. serotype H-14, a suspension was prepared by adding 1 ml of commercial formulation to 1000 ml distilled water, then prepare required serial concentrations .

In Case of *B.sph* 1593-4, a suspension was prepared by adding 0.25 ml distilled water and serial dilutions were carried out. All bacterial suspensions were vigorously shacked prior to each use and freshly prepared and expressed in ports per million (ppm) in order to calculate the LC_{50}. Plant extract concentrations were prepared by dissolving dry grinding parts in ethyl alcohol and serial concentrations were carried out .

b)Combined effect of bacterial and botanical extract :-

The combined action of the botanical extract and each bacterial larvicides, as well as the effect of the two bacterial preparation together were tested the same technique previously mentioned for the determination of the median lethal levels was followed, instead of using one larvicide , one ml from the concentrations which produced 50% mortality of each larvicide was added to plastic cup containing 98 ml distilled water simultaneously susceptibility experiments of larvae that were pre-treated with the bacteria or the chemical larvicide for 24 hrs and then treated with the order larvicides were carried out in six different combinations in table (1) as follows :-

Table (1):Different combinations of larvicides

Pre- treatment	Additional treatment after 24 hrs
1-*B.t* H-14	*B. sph*
2-*B.t* . H-14	*A. hippocastunum*
3- *B . sph*	*B . t . H-14*
4- B . sph	*A . hippo.*
5- A. hippo.	*B . to H-14*
6- A. hippo.	*B . sph*

Notes

1)*B. t* H – 14 *(Bacillus thuringiensis H-14).*
2)B. sph (Bacillus sphaericus) .
3)A. hippo . (Aesculus hippocastanum)
.

C) Mortality reading:

Mortality date were recorded 24, 48 , 72 hrs after treatment . Percentage of mortalities was corrected by Abbott's formula when the mortality among control experiment exceeds 5%.

d) Statistical analysis of data :-

Percentage of mortality was converted into probits, which were then plotted against the concentrations. Median lethal concentration (LC_{50}) was determined.

T-test was used to evaluate the effect of all deferent larvicides either when they were used separately, in mixtures of two preparations or when one preparation was firstly applied when followed by another different preparation 24 hours later.

3. Results

3. 1- Comparative susceptibility of *Aedes aegyptii* Larvae to the larvicides mixtures.

The larvae mortality resulted from treating the 3rd instar larvae of *Aedes aegypti* with the following mixture of:

***a-* (Bacterial + botanical) larvicides .**
a-1(Bacillus thuringiensis + A. hippo.) mixture .
a-2(Bacillus sphaericus + A. hippo.) mixture .
b. Bacterial mixture
(B- thuringiensis + B. sphaericus)

Mixture were represented in table (2) as follows :-

The data in table (2) indicates that , there is a significant increase in larvae mortality occurred from the use of larvicides mixture(pl0.05) . Comparison between the combined effect of the mixture of (*B.t + A.hippo*.) and the mixture of (*B. sph. + A.hippo*.) indicate that there is no significance

difference between the mortality percent occur to 3 rd instar *A. aegyptii* larvae at 24 and 48 hr exposure tim , but at 72 hr exposure period there is a significance difference show that the (*B.sph* . + *A.hippo*.) mixture is effective ($p<0.05$). Also comparison between the combined effect of the bacterial mixture (*B.t* .+ *B.sph*.)and *(B.t* . +*A. hippo*). Mixture indicate that the first mixture have a significant effect that the second mixture ($p<0.05$), while , the bacterial mixture (*B.t* + *B. sph*.) have highly significant effect than (*B. sph* + *A. hippo*.) mixture ($p<0.05$) .

Table (2) : Percentage mortality of the early 3rd instar Larvae of *Aedes aegyptii* treated with mixtures of (*B.t* + *A.hippo.) , (B.sph.+A.hippo) or (B.t + B. sph.).*

Average of corrected mortality percent			
Combined mixture of LC50s of the tested larvicides	**Exposure time**		
	24 hrs	**48 hrs**	**72 hrs**
(*B. t* H-14 + *A. hippo*) 0.037 ppm + 0.013 ppm	**X ± SE**	**X ± SE**	**X ± SE**
	70.80 ± 0.28	**81.04 ± 0.56**	**88.32 ± 0.44**
(*B. sph.* + *A. hippo* 2.6 ppm + 0.013 ppm)	**69.50 ± 0.83**	**85.84 ± 0.97**	**94.30 ± 0.78**
(*B.t* H-14+*B. sph*) 0.037 ppm + 2.6 ppm	**78.82 ± 0.08**	**96.91 ± 0.34**	**100**

2. Effect of using the bacterial and botanical Larvicides on *Aedes aegyptii* Larvae in sequence:

The susceptibility of the 3rd instar larvae of *A. aegyptii* to the toxic effect of the entomopathogenic bacteria , *Bt*. H-14 or *B. sph* when pre-treating the larvae with it for 24 hrs and then the other bacteria or *A. hippo*. was added, were evaluated through carring out a series of laboratory experiments. The results obtained in table (3) indicated that, adding *B. sph*. To larvae pre-treated with *Bt*. for 24 hrs cause significant mortality than if *B. sph*. was used alone also , adding *A. hippo*. Resulted in a significant larval mortality than if botanical was used alone at first 24 hrs , but with increasing exposure time no significant mortality occur when adding *B. sph.*

Treating the 3rd instar of *A.aegyptii* larvae with *B. sph*. For 24 hrs with LC_{50} (2.6 PPm) then *Bt* H-14 or *A.hippo*. larvicides was added in order to evaluate its effectiveness were represented in table (4) and indicating that , pretreating larvae with *B. sph*. For 24 hrs then *Bt*. was added cause a significant increase in larval mortality than if *Bt* was used alone.

Table(3) : Percentage mortality of the early 3rd instar larvae of *Aedes aegyptii* pre-treated with *Bt*. H-14 , LC_{50} (0.037 PPm)for 24 hrs to *B. sph* , LC_{50}(2.6 PPm) or *A. hippo.* , LC_{50}(0.013 PPm).

Post-Exposure time	***B. sph.***		***A. hippo***		**Average of corrected mortality percent.**	
	LC_{50} ppm	**LC_{90} ppm**	**LC_{50} ppm**	**LC_{90} ppm**	**Larvae pre-treated with *B. t.* for the first 24 hr with LC_{50} conc. = 0.037 ppm**	
24 hrs	**2.6**	**8**	**0.013**	**0.027**	**Larvae post-mixed treatment with B. sph**	**Larvae post-mixed treatment with *A.hippo*..**
					X ± SE	**X ± SE**
					77.30 ± 0.05	**88.18 ± 0.39**
46 hrs	**1.18**	**2.7**	**0.011**	**0.022**	**99.99 ± 0.01**	**96.12 ± 0.98**

Table (4): Percentage mortality of the early 3rd istar larvae of *Aedes aegyptii* pre-treated with *B. sph*. 1593-4 , LC_{50}(2.6 ppm) for 24 hrs to *B.t* ,LC_{50}(0.037ppm) or *A. hippo*. LC_{50}(0.013ppm).

Post-Exposure time	**B. t. i.**		***A. hippo***		**Average of corrected mortality percent.**	
	LC_{50} PPm	**LC_{90} PPm**	**LC_{50} PPm**	**LC_{90} PPm**	**Larvae pre-treated with *B. sph.* for the first 24 hr with LC_{50} conc. = 2.6 PPm**	
24 hrs	**0.037**	**0.15**	**0.013**	**0.027**	**Larvae post-mixed treatment with *B. t.* H-14**	**Larvae post-mixed treatment with *A. hippo***
					X ± SE	**X ± SE**
					90.02 ± 0.35	**85.95 ± 0.21**
46 hrs	**0.022**	**0.1**	**0.011**	**0.022**	**100**	**99.01 ± 0.30**

3. Effect of pre-treating *Aedes aegyptii* larvae with *A. hippo*. for 24 hrs then bacterial larvicides was added .

Finding the potential efficacy of *A. hippo*. Larvicide when the tested larvae were pretreated with it for 24 hrs with the LC_{50} (0.013PPm) and then *B. t*. or *B. sph*. was added , were done through carrying out a series of laboratory experiments and the results were tabulated in table (5) . The results indicated that , pretreatment with *A. hippo.* Then adding *B.t*. or *B.sph* cause a significant increase in larval mortality.

Table (5) : Percentage mortality of early 3rd isstar larvae of *A. aegyptii* pre-treated with *A.hippo* LC_{50} (0.013ppm) for 24 hrs to B. t. H-14 , LC_{50} (0.037ppm)or *B.sph*. 1593-4 , LC_{50}(2.6ppm)

Post-Exposure time	*B. t* H-14		*B. sph.*		Average of corrected mortality percent.	
	LC_{50} ppm	LC_{90} ppm	LC_{50} ppm	LC_{90} ppm	Larvae pre-treated with *B. t.* for the first 24 hr with LC_{50} conc. = 0.037 PPm	
					Larvae post-mixed treatment with B. t H-14.	Larvae post-mixed treatment with *B.sph.*
					X ± SE	X ± SE
24 hr	0.037	0.15	2.6	8	92.11 ± 0.10	88.99 ± 0.31
46 hr	0.022	0.1	1.18	2.7	97.26 ± 0.04	98.87 ± 0.40

4- Comparative susceptibility of *Aedes aegyptii* larvicide mixture when used in sequence.

The results of pre-treating the *A. aegyptii* larvae for 24 hrs with bacterial larvicides and then the other botanical or bacterial larvicides was added and vice-versa was represented in table (6) as follows :-

No significant differences occurred to larval mortality rates when the tested larvae was pretreated with the two bacterial biocides and then *A. hippo* was added , ($p<0.05$). Mortality occurred as a result of pretreating larvae with *B.sph*. Followed by *B.t*. was found to be significant ($p<0.05$). while no significant difference was obtained as treatment with *A. hippo*. And followed by *B. sph*. or *B. t*. ($p<0.05$).

Table (6): Comparative susceptibility of early 3rd *Aedes aegyptii* larvae pre-treated with *A. hippo., B.t.* H-14 or *B.sph*. 1593-4 for 24 hrs . then the other larvicides were added .

Larvae pre-treated for 24 hrs with	The added larvicide	Averages of corrected mortality percent	
		Exposure time	
		24 hrs	48 hrs
B. thuringiensis Serotype H-14	*B. Sphaericus* *A.hippo*.	X ± SE	X ± SE
		77.30 ± 0.05	99.99 ± 0.01
		88.95 ± 0.39	96.12 ± 0.98
B. Sphaericus 1593-4	*Bti* H-14	90.02 ± 0.35	100
	A.hippo	85.95 ± 0.21	99.01 ± 0.40
A. hippo	Bti H-14	92.11 ± 0.10	97.26 ± 0.04
	B. Sphaericus	88.99 ± 0.31	98.87 ± 0.40

4. Discussion and conclusion

When toxins from B.t. subsp. *Israelensis* or B. *sphaericus* were combined with a botanical extract of *A. hippocastanum* synergestic interactions increased toxicity and expanded the effect range of the mixtures. where as synergy is well documented among the native endotoxins of *B. t.* subsp. *Israelensis* , it is evident from this and earlier studies with cytolytic toxins that synergy is not limited to native toxins combinations but can occur between toxins from unrelated bacterial strains , i. e. between toxins of *B. spharaericus* , *B. t i*. or botanical extract.

Mixtures of *B. sph.* , *B. t. i.* were very active against the normally insensitive mosquito species *Ae. Aegypti* . Toxicity values also were generally not significantly different from those of *A. hippocastanum* those same mixtures showed improved activity against susceptible *A. aegytii* , demonstrating that activity improvement can be achieved toward normally sensitive mosquito as well . That activity is due in part to the inherent toxicity of B. t. i. toxins. however, additional

activity must be attributed to the synergism of *B. sphaericus* (Wirth *et al*., 2000 a, b, c) and synergy involving bacterial toxins . Whether that activity well translate into improved field toxicity remains to be seen .

The broad spectrum of synergy that is now apparent suggests that complex interaction occur among most toxins in *B. t. i. , B. sphaericus* and are probably responsible for much of the increased toxicity (Bourgouin *et al*., 1990, Doncel *et al.* , 1997, Thiery *et al*., 1998, Servant *et al*., 1999 , Li *et al*., 2000 , Sun *et al*., 2001 , Zahiri *et al*., 2002 .

These interaction should provide some level of protection against insecticide resistance because they involve toxins that target different receptors in mosquito midgut (Silva – Filha *et al*., 1999) interact synergistically , traits that naturally occur in *B. t. i.* and B.t. jegathesan (Selena *etal*.,1995) and are believed to retard the evolution of resistance to those two bacterial species (Geoeghiou and Wirth, 1997, Wirth *et al*., 2004) . Furthermore , a mixture of *B. t.* and *B. sph*. Was experimentally demonstrated to delay *B. sph*. Resistance (Zahiri and Mulla, 2003 , Abozinadah *et al*., 2011 and Abuldahab *et al*., 2011 .

B. sphaericus , because of its high activity in polluted water and long residual activity , has an important role in mosquito larval control that is at risk because of its limited host range and propensity to select resistance the risk for resistance potentially reduced by combinating *B. sphaericus* with toxins from B. t. i and active groups of botanical extract (Yulin *et al*., 2011). Botanicals also used as biological control agents due to its ability to reduce mosquito resistance and as a safe method against the target insect (Abuldahab and Younes,1999; Gusmao *et al*. , 2002 Cavados *et al.* 2004 , Amusan *et al.* 2005 , Abdel Halem, 2006 and Nalhan, 2007). Based on the experimental results of bioassaying each of the three tested larvicides individually or in combinations , as well as on studying their pathological action, it may be assumed that it is preferable to use these biological larvicides in integration with the chemical one in mosquito control programs particularly in larval control , instead of using each of them alone .

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