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Full Length Research Paper

Biotoxicity assay of *Bacillus thuringiensis* combined with sodium citrate, Bifenthrin+Cypermethrin and *Saraca indica* on *Tribolium castaneum*

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Microbial control of insect pest of crops using entomopathogens is pest management strategy. *Bacillus thuringiensis* has a great potential for this purpose. The focus of the present study was to determine the individual and combined effect of different insecticides such as *Saraca indica* as a botanical insecticide, *B. thuringiensis* and chemical insecticides against larvae and adults of *T. castaneum*. Results were analyzed by SPSS probit analysis, LC_{50} was noted for all these insecticides and results were compared with each other. According to the results from the individual insecticide *B. thuringiensis* show high mortality with harmless effect to environment. Synergistic effect with high mortality rate was observed at 24 to 48 h, for both larvae and adults of *T. castaneum.* From the study it was observed that mortality rate increases as the concentrations increases, and larvae show higher mortality because they were more susceptible to these insecticides as compared to adults. Percentage mortality of *T. castaneum* in combined bioassay sodium citrate, *S. indica, B. thuringiensis* and Bifenthrin+cypermethrin was compared with the mortality of individual insecticides. 61% larvae and 55% adult died in combine bioassay at high concentration.

Key words: *Bacillus thuringiensis, Saraca indica, Tribolium castaneum,* Bifenthrin, Cypermethrin, biotoxicity assay.

INTRODUCTION

Tribolium castaneum is known as "bran bugs" and it commonly attacks stored grain products, such as cereals and flour. Both larvae and adults of red flour beetles feed on broken kernels and grain dust. These stored pests are mostly get rid of in the house in infected cereal or flour while some red flour beetles survive on food material in, crevices, furniture and cabinet cracks and can increase their descendants (Haque et al., 2000). *T. castaneum* breed in grain dust, damaged grain, flour, elevated-humidity flour essences, etc. Each feminine beetle laid three hundred to four hundred spawn in wheat or other food dust throughout a time phase of five to nine months. After five to nine month larval period start. During one or two week, these offspring emerge into circular, larvae with creamy color .

Bacillus thuringiensis is a naturally occurring insecticide. As this bacteria is the active ingredients of some pesticides this remarkable distinctive quality facilitate its functional for insect management. *B. thuringiensis* insecticides are most commonly used against some larvae, adult and caterpillars. *B. thuringiensis* is considered as safe to people and non target species,

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such as mammals and wildlife. Some *B. thuringiensis* formulations can be used on essentially all food crops, because different types of Cry proteins are present in it, which are effective for different orders of insects (Jilani and Ahmad, 1982). *B. thuringiensis* contain valuable environment-proteins of Cry genes. So it is considered as friendly bio pesticide, which constitutes 90% of the world significance by the production of insect resistant crops, such bio pesticide commercially. Its insecticidal properties were analyzed against maize, cotton, potato, rice, etc (Kumar, 2002). *B. thuringiensis* is Gram positive spore forming aerobic bacterium synthesized during the sporulation phase, isolated from environments like soil. The importance of *B. thuringiensis* is due to its domains. Domain I is made of seven a-helices, domain II accumulates certain proteins in crystalline form and three antiparallel ß sheets, are formed during the sporulation phase (Kumar and Bambawale, 2002). These sheets are folded into loops. Domain III is made of ß proteins which are very toxic towards larvae of different orders of insect pests as Lepidoptera, Diptera, Coleoptera. While a set of two antiparallel ß strands show effect response against Hymenoptera, and Homoptera with different efficacies (Schnepf et al., 1998). Domain II is hyper variable in nature which determines the insecticidal activity. More than 150 different Cry toxins have been cloned and specificity of a toxin is determined against different insets. Test for the toxicity of Domain III on various insect species is still on till date (Crickmore et al., 1998). *B. thuringiensis* and its d-endotoxins have been extensively studied for their molecular mode of action and con-struction of novel toxins with enhanced molecular mode of action and toxin structural-function insecticidal activity and specificity relationships. Focus of this study was to analyze how protein engineering of d-endotoxins of different domains helps in death of target insect. Structure of *B. thuringiensis* d-endotoxin Cry3Aa describing the three domains (Li et al., 1991).

Botanical insecticides are most widely used in pest control and mostare unique in action, safe to apply, and can be easily provided for usage. Locally available plants and minerals have a broad spectrum in protecting stored products against damages by insects' infections. The main advantage of botanicals is that they are easily provided on small-scale industries produced by farmers, and are mostly less expensive. Herbal medicine is the most ancient form of health care for humans. Most of the prescript drugs are tree derivatives, herbs, or shrubs, and are known as medicinal plants (Chapman, 1974). *Saraca indica* L. is a small ever green tree. It contains a large number of naturally occuring chemical that have biological activity. It is grown in all over Pakistan (Rasool et al., 1991).

The widespread use of insecticides has given rise to many biochemical as well as physiological changes, to the life of an animal. This may be of adaptive significance for human beings. Sodium citrate is a granular white crystalline or, water soluble, odorless solid, having a cool saline taste (Doane and Wallis, 1964).

Manufactured imitative of usually occurrence pyrethrins, may be known as synthetic pyrethroids, which are in use from pyrethrin, taken out from dehydrated plant of chrysanthemum (for natural pyrethrins or synthetic pyrethroids a generic term “pyrethrum” is used). The insecticidal qualities of pyrethroids may be determined from ketoalcoholic ions of pyrethroic acids in addition to chrysanthemic. Mode of action of these insecticides is that these acids are strongly lipophilic and paralyze the nervous system of target insect rapidly (Soderlund et al., 2002).

In recent years, controlling stored insect pests have been most commonly performed by synthetic insecticides; although alternative methods have decrease the insecticides usage to reduce human contact potential in addition to diminish pest resistance to pesticides (Aldryhim, 1993).

MATERIALS AND METHODS

Insect rearing

Twenty individual mating pairs were maintained in glass jars containing 250 g of diet (samolina and 10% yeast extract) and covered with muslin cloth, third instar larvae and newly emerged adults were taken from the culture and were used in Bioassays.

Sample collection of *B. thuringiensis*

300 samples of organically rich soil, animal dung, bird droppings and grain dust were collected from different areas of Lahore in sterile and properly labeled glass jars.

Sample processing technique for the isolation of *B. thuringiensis*

Samples collected from different habitats of Lahore were processed for the isolation of *B. thuringiensis* according to Martin and Travers (1989). Briefly, samples (0.5 g) were suspended in 10 ml of LB medium (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) containing 0.2 M sodium acetate, shaken well and incubated at 30°C for 4 h. The incubated samples were filtered using filter paper (0.25 nm) and heated at 80°C for 15 min to isolate spore formers. The above treated samples were diluted 1:2 and then spread on LB agar plates and incubated overnight at 30°C.

Screening of *B. thuringiensis*

The growth of *B. thuringiensis* best occurs in LB medium so LB medium was used in order to study the colony morphology of the isolates. After 24 h, the growth on LB-agar plates was observed and those colonies were selected which contained morphology apparently like that of *B. thuringiensis* (entire margin, off white color, dry and rich growth of colony) were picked and streaked on LB agar plates and incubated overnight at 30°C for 24 h. A single colony was picked from the pure culture for smear preparation then Gram staining and spore staining was performed for further identification of *B. thuringiensis*. After confirmatory test of *B. thurringiensis,* it was grown on petriplates of T3 medium (Table 1).

Table 1. Morphological characteristics of *Bacillus thuringiensis* observed by Gram staining and endospore staining.

Sample	Gram reaction	Cell arrangement	Endospore staining	
Soil sample	By staining smear retain crystal violet and appeared as deep violet	Single, Rod shaped Bt was examined	Vegetative cells	Spore
		Chains of rod shaped Bt was also appeared as deep violet in color	Pink	Green

T3 Media preparation

T3 medium was prepared by adding 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.0005 g of MnCl 2.5 ml of 1 M Potassium phosphate (pH = 6.9) and 15 g of agar/liter.

Streaking

When the plates were solidify streaking of SD1 strain was done with the help of inoculating loop and plates were placed in incubator for 72 h. After 72 h the growth was collected with the help of the inoculating loop in the falcon tube then distilled water was added to the falcon tube and shaken it well. A suspension was made after it was added to the centrifuge tube and centrifuged at 300 rpm to obtain the pure *B. thuringiensis* pellet for the bioassay. From all 300 samples the most toxic SD1was selected for bioassay.

Bioassay

Control

1 g diet (0.8 g of Samolina and 0.2 g of yeast extract) was added in the glass vial. Sixty larvae were added in the glass vials in triplicate form and labeled as control. In the same way 1 g of diet was added in three other glass vials and 20 newly emerged adults were added in each vial containing diet as a control.

Bioassay of *B. thuringiensis*

Three concentrations of 0.5, 1.0, 1.5 g of *B. thuringiensis* pellet were added in each vial containing one gram diet. Twenty larvae of *T. castaneum* were placed in each replicate and mortality was observed after 24, 48, and 72 h. The same concentrations were made for newly emerged adults of *T. castaneum* in separate glass vials. Bioassay of adults was also done in triplicate results were observed after every 24, 48 and 72 h at 30°C.

Sample collection of *S. indica*

Fresh leaves of *S. indica* were collected from Lawrence garden Lahore and dried separately at room temperature under shed for 10 to 12 days, to avoid vaporization of their volatile compounds. After 12 days leaves were completely dried, and then they were grinded to make leave powder.

Bioassay of *S. indica*

Three concentrations of 1.5, 2.0, and 2.5 g of *S. indica* powder were added in each vial containing diet. Twenty larvae of *T. castaneum* were placed in each vial of the same concentration and results were observed at daily bases for 3 weeks.

Same concentrations were made for newly emerged adults of *T. castaneum* in separate glass vials. Bioassay of adults was also done in triplicate form and results were observed daily till 3 weeks.

Bioassay of sodium citrate

1 g diet (0.8 g of Samolina and 0.2 g of yeast extract) was added in each glass vial. Three concentrations of 1.0, 1.5, 2.0 g of sodium citrate were added in each vial containing diet. Twenty larvae of *T. castaneum* were placed in each vial of the same concentration. Results were observed at 24, 48, and 72 h. The same concentrations were made for newly emerged adults of *T. castaneum* in separate glass vials. Bioassay of adults was also done in triplicate form and results were observed at 24, 48 and 72 h.

Bioassay of Insecticide Bifenthrin+cypermethrin

0.05% of insecticide Bifenthrin+cypermethrin were made by taking 0.00001 ml of insecticide in 10ml of acetone. One ml of this diluted solution was rinsed in each vial and allow it to evaporate and vials become dry than 1 g diet (0.8 g of Samolina and 0.2 g of Yeast extract) was added in the rinsed vials. In the same way 0.15, 0.25% of insecticide Bifenthrin+cypermethrin were made by taking 0.00004 ml and 0.00006 ml in 10 ml of acetone respectively. Sixty larvae of *T. castaneum* were placed in the vials rinsed with insecticide and containing 1 g diet in triplicate form as 20, 20, 20 larvae in each vial of the same concentration. And results were observed at 24, 48, and 72 h.

Same concentrations were made for newly emerged adults of *T. castaneum* in separate glass vials. Bioassay of adults was also done in triplicate form in the same way as in case of larvae, and results were observed at 24, 48, and 72 h.

Combined bioassay of botanical, chemical, and biological insecticide

The combine bioassay was done in three concentrations by adding first, second, and third concentrations of each insecticides. Twenty larvae were added in each vial containing diet in triplicate. Mortality was noted after 24, 48, and 72 h. Same concentrations were applied for newly emerged adults of *T. castaneum* in separate glass vials. Bioassay of adults was also done in triplicate in the same way as in case of larvae, and results were observed after 24, 48, and 72 h.

Statistical analysis

Mortality, LC_{50} of adults and larvae was determined by Probit analysis using SPSS version 13, and regression equation was found using Microsoft Excel.

RESULTS AND DISCUSSION

The present study was planned to check the individual and combine effect of botanical insecticide such as *S.*

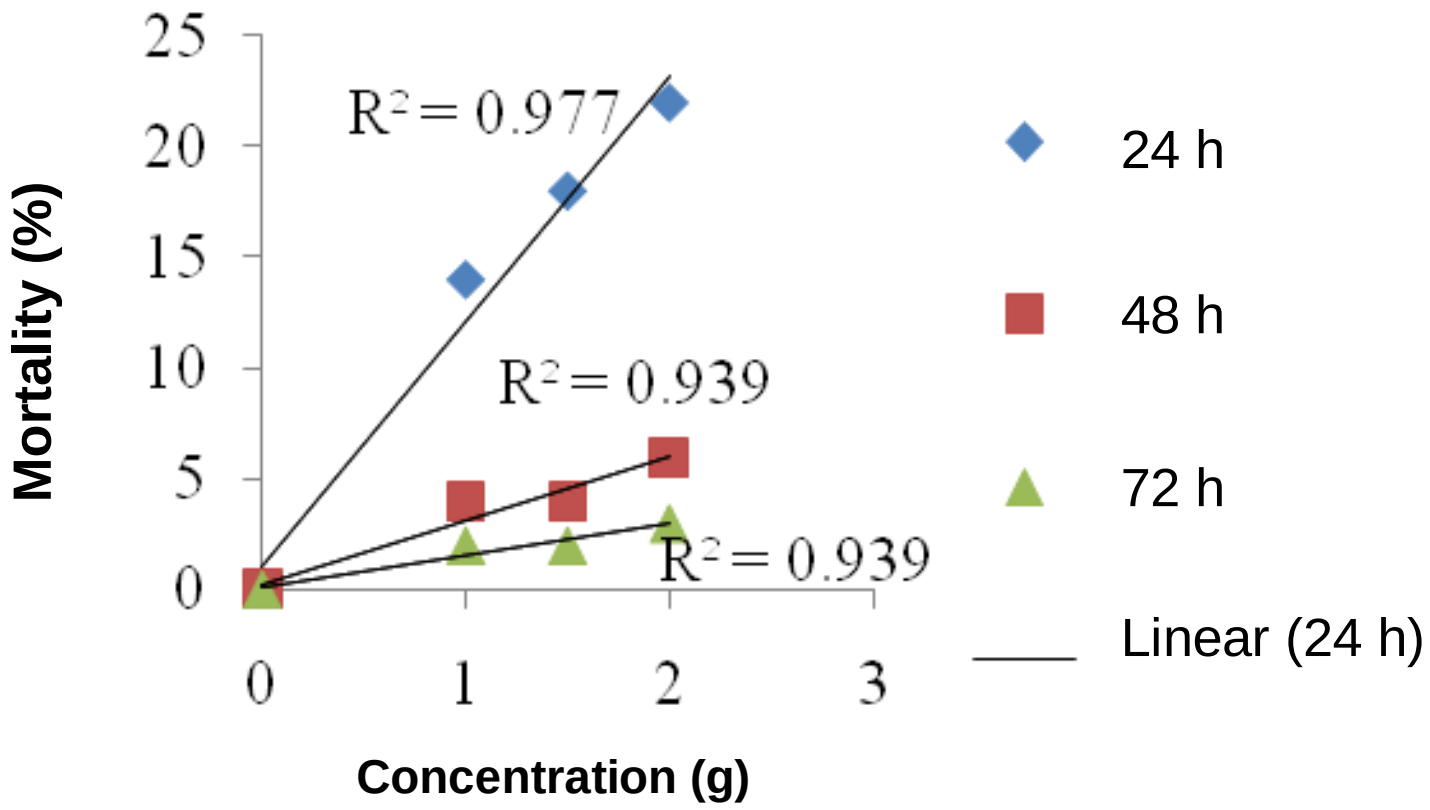

Figure 1. Insecticidal Effect of different concentrations of sodium citrate on T. *castaneum* larvae at 24, 48, and 72 h.

indica, chemical Sodium citratrate, *B. thuringiensis* SD1 strain and insecticide Bifenthrin+cypermethrin on *T. castaneum* larvae and adults (Figures 1 and 2).

Bioassay of Cypermethrin+Bifenthrin on *T. castaneum* larvae and adults

After 24 h, 12, 22 and 30% larvae were died at 0.05, 0.15 and 0.25 g concentration, respectively with LC_{50} value of 0.2 g/g. Results after 48 hours show low mortality than 24 h with LC_{50} 0.35 g/g .While at 72 h LC_{50} 0.36 g/g was observed. Control group showed no mortality, in the same way results were observed for adults.

Bioassay of *S. indica* on *T. castaneum* larvae and adults

After 1 week 12, 14 and 18% larvae were died at 1.5, 2.0 and 2.5 g concentration, respectively with LC_{50} 3.1 g/g. Results after 2 weeks show low mortality than 1 week with 4.5 LC_{50}. While at 3rd week 0.8 g/g LC_{50} was noted because no mortality was observed after 3 week.

According to the present study five groups of insecticides were categorized to check the individual and combine effect of all these biological and chemical insecticide on *T. castaneum* larvae and adults. The present result is the same to Mondal and Akhtar (1994) who stated insecticidal effect of eleven plant materials including neem oil against *Tribolium* beetles. $LC_{50,}$ regression values and percentage mortality were observed in the present study. From the study it was observed that high concentration of each insecticide show high mortality and mortality rate decreases with the passage of time. A study was made by Hussaini et al. (2005) to check the toxicities of some novel insecticides (abamectin, Spinosad, indoxacarb, azadirachtin, buprofezin, and Tenekil 100EC), against two larval strain of *T. castaneum* such as malathion–resistant (PAK) and organophosphate–susceptible (FSS–II). The results indicated that abamectin was the most toxic of all insecticides tested in this study as compared to indoxacarb, spinosad, buprofezin, Tenekil 100EC, and azadirachtin. Furthermore, for the larvae of PAK strain abamectin, spinosad, and buprofezin proved more toxic and indoxacarb, Tenekil, azadirachtin, and 100EC to larvae of FSS-II strain. Abamectin resistance was also noted in field populations of diamondback moth, *Plutella xylostella* (L) (Iqbal et al., 1996; Iqbal and Wright, 1997; Sayyed et al., 2004), when it was compared with a laboratory insecticide susceptible population. The present

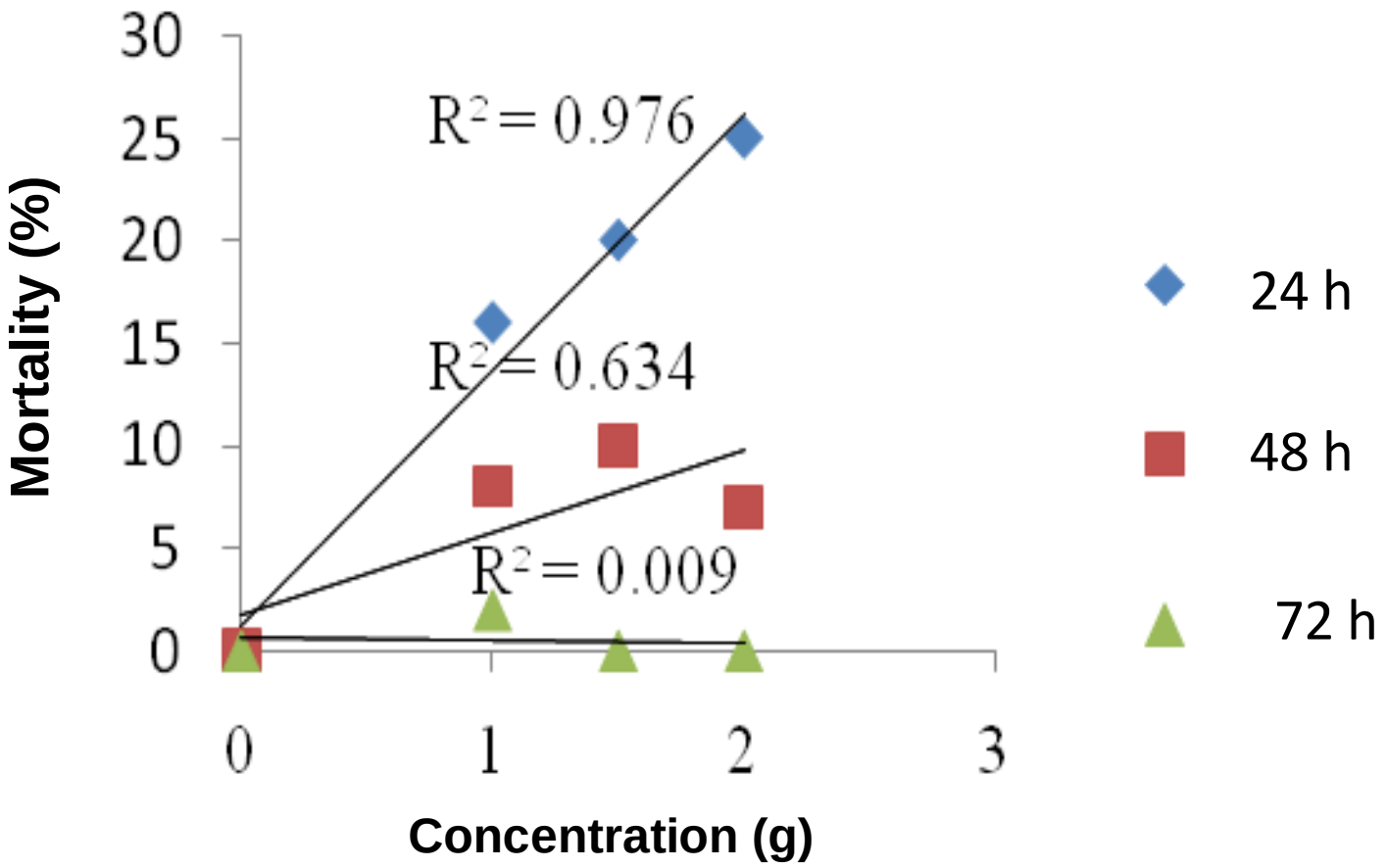

Figure 2. Insecticidal Effect of different concentrations of *Bacillus thuringiensis* SD1 strain on newly emerged adults of *T. castaneum* at 24, 48, and 72 h.

study was done to check the synergistic effect of different biological and commercial insecticide on *T. castaneum.* Classification of synergism was presented by Benz (1971). According to him, there is supplemental synergism in chemical and bacteria. A system of two effective components such as effect of *B. thuringiensis* and boric acid or sodium citrate can be increased as it together produce greater effect than algebraic sum of the individual effect. In the present studies it was seen that a combination of *B. thuringiensis* and boric acid was more effective in causing the death of the termites. The combine effect of 1% boric acid and *B. thuringiensis* may be considered as supplemental synergism. Toxicity of *B. thuringiensis* was enhanced by the chemical sodium citrate and very high mortality was observed in case of combine bioassay of larvae and adults. As *B. thuringiensis* easily degradable so after 72 h it becomes degraded, and half life of Cypermethrin+Bifenthrin 3.6% EC is also just about 72 h. At 24 h all these insecticides are more active and very high mortality was observed at 24 h on all three concentrations, 61% larval mortality and 55% mortality of adult were observed at high concentration (Table 2 and 3).

Conclusion

From the study it was concluded that combined insecticides showed high mortality than individual insecticides. Many insecticides were used as; chemical insecticides, *B. thuringiensis* formulation, and botanical insecticide against *T. castaneum* larvae and adults. It was observed that *B. thuringiensis* formulation show very effective result as compared to others with no residual effect, although botanical insecticide (*S. indica*) was also eco friendly but slow in action and low mortality was observed due to repellency and chemical insecticide (Cypermethrin+Bifenthrin) show residual effect to environment. While in combine bioassay synergistic effect with high mortality rate was observed as compared to all individual insecticides, with harm less effect to environment. Many *B. thuringiensis* based bio pesticides are formulated in recent years against food pests. From the study it may concluded that a bio pesticide can be made by combination of all these insecticides to control many pests. This will be environment safe having capacity to kill the target organism more rapidly with no harm full effect to non target organisms.

Table 2. Comparison of individual and combine effect of sodium citrate, S. *indica*, *B. thuringiensis* SD1 strain and Cypermethrin+Bifenthrin, on *T. castaneum* larvae at 24, 48 and 72 h.

Time	Interval	Combine and individual concentration mixed with diet		Observed mortality D/T	Percentage mortality (%)
24	First concentration	Control		0/60	0
		Sodium citrate	1.0 g	14/60	23.3
		B. thuringiensis	1.0 g	20/60	33.3
		S. indica	1.5 g	12/60	20
		Bif+Cyp	0.05%	12/60	20
		Combine		20/60	33.3
48		Control		0/60	0
		Sodium citrate		4/60	6.6
		B. thuringiensis		7/60	11.6
		S .indica		6/60	10
		Bif+Cyp		10/60	16.6
		Combine		13/60	21.6
72		Control		0/60	0
		Sodium citrate		2/60	3.3
		B. thuringiensis		1/60	1.6
		S. indica		0/60	0
		Bif+Cyp		8/60	13.3
		Combine		2/60	3.3
24	Second concentration	Control		0/60	0
		Sodium citrate	1.5 g	18/60	30
		B. thuringiensis	1.5 g	23/60	38.3
		S. indica	2.0 g	14/60	23.3
		Bif+Cyp	0.15%	22/60	36.6
		Combine		25/60	41.6
48		Control		0/60	0
		Sodium citrate		4/60	6.6
		B. thuringiensis		10/60	16.6
		S. indica		5/60	8.3
		Bif+Cyp		8/60	13.3
		Combine		14/60	23.3
72		Control		0/60	0
		Sodium citrate		2/60	3.3
		B. thuringiensis		0/60	0
		S. indica		1/60	1.6
		Bif+Cyp		7/60	11.6
		Combine		1/60	1.6
24	Third concentration	Control		0/60	0
		Sodium citrate	2.0 g	22/60	36.6
		B. thuringiensis	2.0 g	27/60	45
		*S. ind*ica	2.5 g	18/60	30
		Bif+Cyp	0.25%	30/60	50
		Combine		37/60	61

Table 2. Contd.

48	Third concentration	Control	0/60	0%
		Sodium citrate	6/60	10
		B. thuringiensis	5/60	8.3
		S. indica	5/60	8.3
		Bif+Cyp	8/60	13.3
		Combine	15/60	25
72		Control	0/60	0
		Sodium citrate	3/60	5
		B. thuringiensis	0/60	0
		S. indica	0/60	0
		Bif+Cyp	5/60	8.3
		Combine	0/60	0

Table 3. Comparison of individual and combine effect of sodium citrate, *Saraca indica. Bacillus thuringiensis* SD1 strain and Cypermethrin+Bifenthrin on *Tribolium castaneum* newly emerged adults at 24, 48 and 72 h.

Time (h)	Interval	Combine and individual concentration mixed with diet		Observed mortality D/T	Percentage mortality (%)
24	First concentration	Control		0/60	0
		Sodium citrate	1.0g	8/60	13.3
		B. thuringiensis	1.0g	16/60	26.6
		S. indica	1.5g	9/60	15
		Bif+Cyp	0.05%	10/60	16.6
		Combine		20/60	33.3
48		Control		0/60	0
		Sodium citrate		5/60	8.3
		B. thuringiensis		8/60	13.3
		S. indica		5/60	8.3
		Bif+Cyp		8/60	13.3
		Combine		10/60	16.6
72		Control		0/60	0
		Sodium citrate		5/60	8.3
		B. thuringiensis		2/60	3.3
		S.indica		1/60	1.6
		Bif+Cyp		7/60	11.6
		Combine		0/60	0
24	Second concentration	Control		0/60	0
		Sodium citrate	1.5g	10/60	16.6
		B. thuringiensis	1.5g	20/60	33.3
		S. indica	2.0g	12/60	20
		Bif+Cyp	0.15%	15/60	25
		Combine		25/60	41.6
48		Control		0/60	0
		Sodium citrate		6/60	0.1
		B. thuringiensis		10/60	16.6
		S. indica		6/60	0.1
		Bif+Cyp		10/60	16.6
		Combine		12/60	20

Table 3. Contd.

72	Control	0/60	0
	Sodium citrate	4/60	6.6
	B. thuringiensis	0/60	0
	S. indica	1/60	1.6
	Bif+Cyp	8/60	13.3
	Combine	1/60	1.6

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