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Efficiency of *Bacillus thuringiensis* var *krustaki* with Henna essence and Neemarin (Neem toxicant - *Azadirachta indica* A.) on second and third instars larvae cabbage large white butterfly *Pieris brassicae* L. (Lep: Pieridae) in Lab conditions

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Abstract
Cabbage large white butterfly *Pieris brassicae* L. is one of the most important pests of cabbage crops in Urmia city of west Azerbaijan province. According to risks of pesticide residue and environmental damage using biological control agents, especially *Bacillus thuringiensis* is considered. In the current research, the effect of *B. thuringiensis*, Neemarin and Henna essence in separately and combination form on second and third instars larvae was investigated. The result show that combined effects three agents on second and third instars larval stages was additive. Percentage of mortality mixture of bacteria, Neemarin and Henna treatments was increased in compare bacteria, Neemarin and Henna alone, So that the average mortality combined three agent treatment on 2nd and 3rd instars larvae after 24, 48 and 72 hours were (56.66, 63.33 and 66.66%) and (50, 56.66 and 60%), respectively. Our study indicated that the integrating *B. thuringiensis*, Neemarin and Henna as environmentally acceptable agents has opened further opportunities for their uses as integrated management of this pest.

Keywords: *Bacillus thuringiensis*, Henna, Neemarin, Additive effect, *Pieris brassicae* L

Introduction
Cabbage (*Brassica oleracea* L.) is an important vegetable crop grown in many countries in the world [36]. The large white *Pieris brassicae* (Lepidoptera: Pieridae) is one of the most serious pests of cruciferous crops in many parts of the world [27]. During the past six decades, chemical preparations have dominated in pest control. This has brought about the pollution of the environmental, danger to humans, and developing resistance against toxicant of insects and mites [35]. The development and use of biodegradable alternative plant protection agents including *Bacillus thuringiensis* Berliner has become a necessity in integrated pest management programmed of all the major crop plants [32]. Special emphasis is being placed on implementing environmentally safe strategies. Commercial formulates based on *B. thuringiensis* may be a good alternative, as they have been used to control other insect pests successfully [9]. *B. thuringiensis* kills many different kinds of insects and has been developed extensively for pest control in a variety of habitats including field crops and controlling insect vectors of human disease, such as mosquitoes [12]. Commercial formulates based on this bacterium have been used for decades to control insect pests as an alternative to chemicals. Such formulates are environmentally friendly, harmless to humans and other vertebrates [5, 16, 22], which occupies 90% of the world biopesticide market [21] and have shown high compatibility with the use of natural enemies [6, 20, 33]. At present, 60 subspecies of *B. thuringiensis* have been identified with strains having different activities within each subspecies and new strains are continuously identified [12]. Several *B. thuringiensis* var. *kurstaki (B.t.k)*-based commercial formulations are used as alternatives to chemical insecticides for the control of lepidopteran pests [30]. Henna (*Lawsonia inermis* L.) is a plant, which grow wild in abandoned areas [26]. It is widely known as cosmetic agent used to color hair, skin and nails [13]. Henna has many traditional and commercial uses, the most common being as a dye for hair, skin and finger nails, as a dye and preservative for leather and cloth, and as an antifungal agent. Its flowers have been used to create perfume since ancient times [3]. In this research, the effects of *B. thuringiensis* var. *kurstaki* and Neemarin (Neem toxicant) and

Henna essence on second and third instars larvae of *P. brassicae* in laboratory condition was evaluated.

Materials and Methods

The present study was carried out in entomology lab of agriculture faculty, plant protection departments of Urmia University in Iran.

Plant rearing

The cabbage seedling (*B. oleracea* var. *capitata*) was planted in the experimental field of the Department of Entomology, Urmia University.

Insects rearing

The eggs of *P. brassica* were collected from the cabbage fields of Nazllo village nearby of Urmia. Newly hatched larvae were fed with fresh cabbage leaves and they were reared under laboratory conditions 23±2 °C, 65±10% RH and photoperiod at 16:8 (L: D) h on fresh cabbage leaves until second and third instars.

Bio – Insecticides

The commercial formulation based on *B. thuringiensis* used in our assay Bac-S® (*B. thuringiensis* var. *kurstaki*, $3^{\times}10^{9}$ UFC/KG, g^{-1}, WP) produced in Spain by Jose Morera B.L. Ptz (Valentia, Spain).

Botanical – Insecticide

The commercial formulation based on Neem used in our assay was Neemarin produced By Biotech international Ltd. (New Delhi, India). Maker factory orders this product for using against damages of Lepidoptera larvae.

Botanical- Essence

The commercial formulation based on Henna used in our assay was Henna Essence produced By Baraij Essence Ltd. (Kashan, Iran). This product was used for human dermal diseases in Iran.

Determine of LC_{50} and LC_{25}

To estimate the LC_{50} and LC_{25} for two larval stages the experiment was performed by using five concentrations of bacteria and distill water as control treatment in three replications for each treatment. Cabbage leaves contaminating with concentrations bacteria with 0.1% tween-20 surfactant prepared by dipping, they were placed into treatment plastic dishes (24×14×8cm). By using head capsule diameter measuring method, 10 larvae were separated and released into each dish. After releasing larvae into the treatment dishes, the dishes were pierced with needle. The mortality of larvae was counted every 24 h for three consecutive days. The same methods were used for estimating the LC_{50} and LC_{25} Neemarin and Henna essence on two larval stages.

The interaction effects between *B. thuringiensis,* Neemarin and Henna essence

After calculating LC_{50} and LC_{25} value for *B. thuringiensis* and Neemarin and Henna essence on second and third instars larvae, combination effects of *B. thuringiensis*, Neemarin and Henna essence on 2rd and 3rd by leaf dipping method in plastic dishes were evaluated. All experiments in completely randomized design in 8 treatments include LC_{50} of *B. thuringiensis*, LC_{50} of Neemarin, LC_{50} of Henna essence, LC_{25} *B. thuringiensis* plus LC_{25} Neemarin, LC_{25} *B. thuringiensis* plus LC_{25} Henna, LC_{25} Henna plus LC_{25} Neemarin, $LC_{16.6}$ *B. thuringiensis* plus $LC_{16.6}$ Henna plus $LC_{16.6}$ Neemarin and distilled water as control in three replications on 30 larvae were conducted. The mortality after 24, 48 and 72 hours were counted.

Data analysis

The LC_{50}, LC_{25} and $LC_{16.6}$ values (with 95% confidence limits) were calculated by using Probit Analysis Statistical Method, mortality data treatments subjected to analysis of variance (One Way ANOVA) and mean separation tests were conducted with Tukey's HSD with SPSS statistical analysis software (Ver. 22).

Results

Bioassay

LC_{50}, LC_{25} and $LC_{16.6}$ of *B. thuringiensis*, Neemarin and Henna on 2rd larval instars:

LC_{50}, LC_{25} and $LC_{16.6}$ of *B. thuringiensis*, Neemarin and Henna on 2rd larval instars in three times were shown in (Table1).

Table 1: Lethal effect on *B. thuringiensis*, Neemarin and Henna on second larvae instar of *P. brassicae*

Insecticide	Total insect	Time (H)	Slope±SE	Chi-square	Lethal concentration		
					$LC_{16.6}$	LC_{25}	LC_{50}
B. thuringiensis	180	24	1.74±4.535	5.31	1288.70	1650.73	2901.48
	180	48	1.57±6.407	13.57	903.70	1096.78	1702.88
	180	72	1.73±6.300	26.54	686.86	828.77	1269.68
Henna	180	24	3.491±0.22	0.06	1.71	2.31	4.54
	180	48	4.051±0.17	0.76	0.98	1.23	1.99
	180	72	4.043±0.17	10.06	0.99	1.14	1.59
Neemarin	180	24	1.12±3.954	3.55	1.10	1.61	3.83
	180	48	4.462±0.10	5.22	0.64	0.87	1.72
	180	72	4.768±0.10	20.75	0.60	0.74	1.16

LC_{50}, LC_{25} and $LC_{16.6}$ of *B. thuringiensis*, Neemarin and Henna on 3rd larval instars

LC_{50}, LC_{25} and $LC_{16.6}$ of *B. thuringiensis*, Neemarin and Henna on 3rd larval instars in three times were shown in (Table 2).

Table 2: Lethal effect on *B. thuringiensis*, Neemarin and Henna essence on third larvae instar of *P. brassicae*

Insecticide	Total insect	Time (H)	Slope±SE	Chi-square	Lethal concentration		
					$LC_{16.6}$	LC_{25}	LC_{50}
B. thuringiensis	180	24	0.97±4.746	1.708	1508.66	1931.32	3389.72
	180	48	1.58±5.919	8.655	984.11	1208.69	1928.66
	180	72	1.30±5.704	23.404	684.73	856.49	1415.12
Henna	180	24	0.24±3.364	0.345	1.88	2.49	4.75
	180	48	0.18±3.898	0.552	1.10	1.37	2.25
	180	72	0.18±3.94	5.073	1.04	1.20	1.68
Neemarin	180	24	3.865±0.13	2.936	1.17	1.74	3.94
	180	48	4.304±0.11	8.145	0.77	1.02	1.94
	180	72	4.629±0.10	17.224	0.66	0.81	1.29

The second ages of pest were susceptible to biological and botanical agent controls. Results showed second larval stages of *P. brassicae* in the case of mortality percentage in same concentration to three agents, Neemarin was lethality in compare with bacteria and Henna.

Combination effects

Second instars

Effects of treatments, LC_{50} *B. thuringiensis*, LC_{50} Neemarin, LC_{50} Henna essence, LC_{25} *B.t.* + LC_{25} Neemarin, LC_{25} *B.t.* + LC_{25} Henna, LC_{25} Neemarin. + LC_{25} Henna, $LC_{16.6}$ *B.t* + $LC_{16.6}$ Neemarin + $LC_{16.6}$ Henna, on second instar larvae was evaluated and counting the percentage mortality of larvae after 24, 48 and 72 hours (figure 1). The results showed that there was a significant difference between treatments with 95% confidence after 24, 48 and 72 hours (df= 7 & 16, P< 0.05, F= 52.114, Sig= 0.001), (df= 7 & 16, P<0.05, F= 59.314, Sig= 0.001), (df= 7 & 16, P< 0.05, F= 66.943, Sig= 0.001), respectively. According to results in three times showed that combined effects of treatments has the highest mortality compared with other treatments (Figure 1).

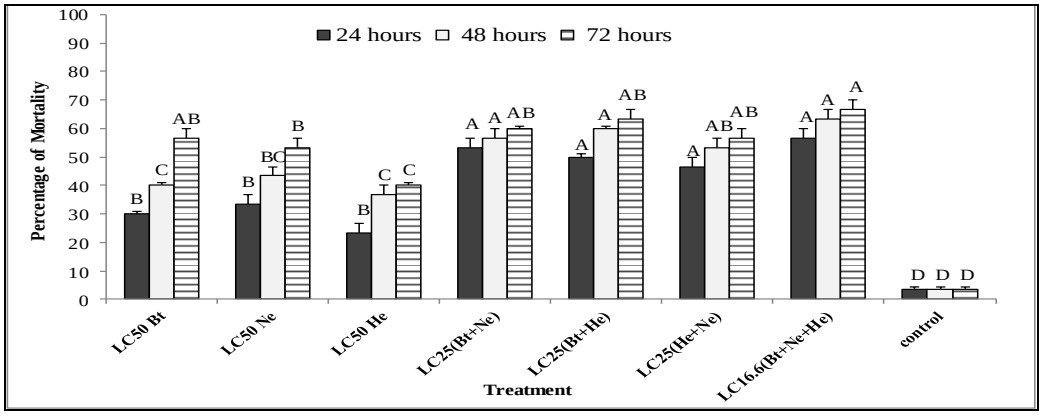

Fig 1: Mortality rate of different treatments on second instar larvae after 24, 48 and 72 hours (Compare mean of treatments in three time independents from each other).

Third instars

Effects of treatments, LC_{50} *B. thuringiensis*, LC_{50} Neemarin, LC_{50} Henna essence, LC_{25} *B.t.*+ LC_{25} Neemarin, LC_{25} *B.t.*+ LC_{25} Henna, LC_{25} Neemarin + LC_{25}Henna, $LC_{16.6}$ *B.t.*+ $LC_{16.6}$ Neemarin + $LC_{16.6}$ Henna, on third instar larvae was evaluated and counting the percentage mortality of larvae after 24, 48 and 72 hours (figure 2). The results showed that there was a significant difference between treatments with 95% confidence after 24, 48 and 72 hours (df= 7 & 16, P< 0.05, F= 42.857, Sig= 0.001), (df= 7 & 16, P<0.05, F= 48, Sig= 0.001), (df= 7 & 16, P< 0.05, F= 67.679, Sig= 0.001), respectively. According to results in three times showed that combined effects of treatments has the highest mortality compared with other treatments same of on second larval stage (Figure 2).

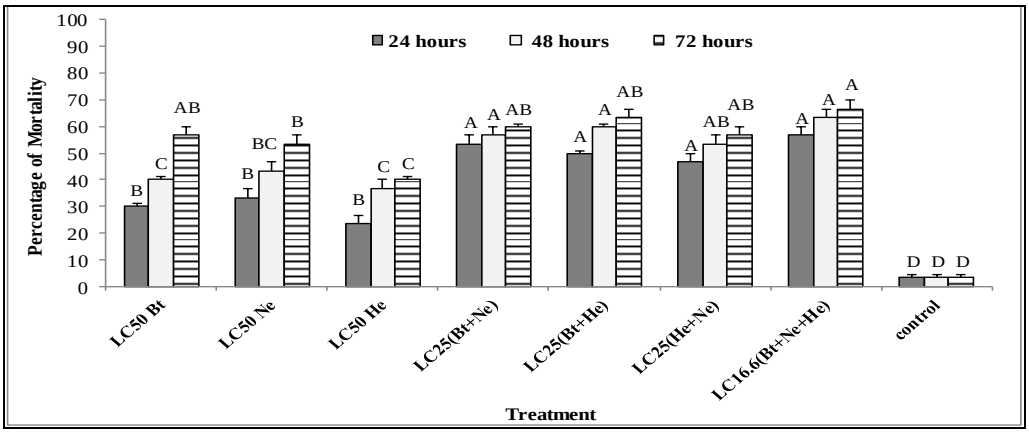

Fig 2: Mortality rate of different treatments on third instar larvae after 24, 48 and 72 hours (Compare mean of treatments in three time independents from each other).

Discussion

A recent survey of biopesticide researchers working in developing countries indicated that formulation was the most important issue in the development of biological insecticides [14]. Microbial pesticides, particularly *B. thuringiensis*, are likely to become increasingly important as pest resistance and environmental concerns reduce the usefulness of conventional insecticides. Although laboratory condition has increased resistance to *B. thuringiensis* in several species of insects [23-25, 34]. Reviewing the previous results it could be concluded that *B. thuringiensis* had potential effect when integrated with Neemarin and Henna that increased the reduction in infestation rates with *P. brassicae* larvae. These results revealed which *B. thuringiensis* var. *kurstaki* used for some Lepidoptera larval control [10]. The results of experiments in this study, showed that *P. brassicae* can be greatly reduced by dipping only *B. thuringiensis*-based formulates, with no need for chemical insecticides, these results supported by Gonzalez -Cabrera *et al.*, [9]. *B. thuringiensis* maintains low toxicity to mammals and other non-target organisms [22]. The laboratory experiments presented in this work are evidence that *B. thuringiensis* is highly efficient in controlling *P. brassicae*. The potential of *B. thuringiensis* formulates in controlling pests of economic importance is well known [31]. Such formulates have been used for years as a key part of Integrated Pest Management programs [9]. *B. thuringiensis* during larval development would be of value in establishing the best management strategies in terms of the timing of biopesticide application.

In our research work results are showed similar efficacy of bio-insecticides *B. thuringiensis* plus Neemarin and Henna essence against *P. brassicae*. The results obtained in laboratory condition that it is possible to reduce the cabbage large white butterfly impact applying biorational insecticide combined with botanical agents. Mixture of *B. thuringiensis* and Neem showed high efficacy in controlling all instar larvae of *Leptinotarsa decemlineata* (Say) giving an average mortality of 60.5% [18]. Danielson [4] found that in all tests, Neem proved to be highly effective against clorado potato beetle larvae. Results revealed Neem was the most effective treatment in restricting the pest infestation Jacobson [17]. These results are similar to those obtained previously in laboratory assays when the highest reduction percentage in the number of larvae was occurred in case of using entomopathogenic bacteria, *B. thuringiensis* combined with botanical extract, Neem [19]. In our study Neemarin were more effective against second and third larval instars of *P. brassicae* alone compared with *B.t.* and Henna and has synergic effects with Bacteria. Henna essence includes many kinds of materials that can be mixed with *B. thuringiensis* and has synergic effect on it. This botanical agent causes injuries in epithelium cells of ileum then Bacteria spores can enter into homocell of insects easily and kill them. Henna that is produced from *L. alba* (Litraceae) possesses several kinds of tannins [1]; and synergistic effects with bacterium [28]. Although the mechanism of interaction between tannins and *B. thuringiensis* toxin is not yet known, it is recognized that tannins could modify the toxicity of *B. thuringiensis* crystal proteins [29]. It is known that tannins act as a toxin and can be used as synergist of biological agents such as *B. thuringiensis*. Henna is due to the large amount of tannins when mixed with bacteria can cause synergistic mode, because the tannins in Henna can cause ulcers in the midgut epithelium cells of insects [2]. This causes the grown spores in stomach to enter homocell and increase larval mortality. Important point is delayed larval mortality time if Henna is used as the synergist. The Henna powder with high mortality rates, delays the mortality of larvae, which also been reported by other researchers [15]. Although deaths caused by a formulation of *B. thuringiensis* plus tannins occurred more slowly than with a high rate of *B. thuringiensis* alone, such formulations would have the advantages of arresting development, minimising foliar damage [8] and might potentially reduce overall *B. thuringiensis* use, thus delaying the development of *B. thuringiensis* resistance [11]. Delay in larval mortality has also been reported by other researchers if tannins are used with bacteria [15]. This subject is related to the chemical properties of tannins where tannic acid reacts with water. The acidity lowers the PH of insects' midgut and decrease the activity of *B. thuringiensis* toxins; therefore, the mortality of insect occurs slowly [7].

In this work, Henna essence mixed with *B. thuringiensis* and Neemarin and results showed this material had additional effects on larvae lethality. The results obtained here from the laboratory condition experiments demonstrated that *B. thuringiensis* effectively controls *Pieris brassicae*. Accordingly, it may be possible to design control programs based on this bacterium that will successfully manage this pest while having low impact on the auxiliary fauna and the environment in general. Our results also show that the Neemarin was more effective in the control of *P. brassicae*, than the *B. thuringiensis* and Henna essence. The combination of *B. thuringiensis*, Neemarin and Henna essence were most effective in the mortality of *P. brassicae* with compared their separate application.

Therefore, further studies should be carried out to integrate this strategy with other integrated or biological control methods in order to reduce the use of chemicals and, consequently, improve food safety and environment quality.

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