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Araştırma Makalesi - Research Article

Insecticidal Activity of Prodigiosin Pigment on *Tenebrio molitor* (Coleoptera: Tenebrionidae)

Prodigiosin Pigmentinin *Tenebrio molitor* (Coleoptera:Tenebrionidae) Üzerindeki İnsektisidal Aktivitesi

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ABSTRACT

Microorganisms and their metabolites have the potential to provide a sustainable alternative to chemical insecticides. *Serratia marcescens* is an entomopathogenic bacterium that produces hydrolytic enzymes and toxins. It also produces a red pigment called prodigiosin, which has a variety of biological properties. In this study, the prodigiosin pigment was extracted from *S. marcescens* Se9 isolate with acidified ethanol and concentrated using a rotary evaporator. The insecticidal potential on larval and adult stages of *Tenebrio molitor* was then investigated. It was found that the mortality rate of larvae exposed to the lowest concentration (125 ppm) of crude pigment was 5%, while the mortality rate of larvae exposed to the highest concentration (2000 ppm) was 68%. The LC_{50} value of the crude pigment for the larval stage was determined to be 924 ppm. On the other hand, even at the highest pigment concentration (2000 ppm), only 30% mortality was observed in adults. The LC_{50} value of the crude pigment for the adult stage was determined to be 4570 ppm. It was determined that the pigment had a toxic effect on the pest, but the larval stage was more sensitive than the adult stage. The study showed that prodigiosin pigment appears to be a promising biocontrol agent for use against *T. molitor*.

Keywords- Serratia marcescens, Bacterial pigment, Prodigiosin, Biocontrol

ÖZ

Mikroorganizmalar ve metabolitleri, kimyasal insektisitlere sürdürülebilir bir alternatif sağlama potansiyeline sahiptir. *Serratia marcescens* hidrolitik enzimler ve toksinler üreten entomopatojenik bir bakteridir. Ayrıca, çeşitli biyolojik özelliklere sahip olan, prodigiosin adı verilen kırmızı bir pigment üretir. Bu çalışmada, prodigiosin pigmenti, asitleştirilmiş etanol ile *S. marcescens* Se9 izolatından ekstrakte edildi ve bir döner buharlaştırıcı kullanılarak konsantre edildi. Daha sonra *Tenebrio molitor*'un larva ve ergin evreleri üzerindeki insektisidal potansiyeli araştırıldı. Ham pigmentin en düşük konsantrasyonuna (125 ppm) maruz kalan larvaların ölüm oranının %5, en yüksek konsantrasyona (2000 ppm) maruz kalan larvaların ölüm oranının ise %68 olduğu tespit edildi. Larva evresi için ham pigmentin LC_{50} değeri 924 ppm olarak belirlendi. Öte yandan, en yüksek pigment konsantrasyonunda (2000 ppm) bile erginlerde sadece %30 ölüm gözlendi. Ham pigmentin ergin evresi için LC_{50} değeri 457 ppm olarak belirlendi. Pigmentin zararlı üzerinde toksik etkisinin olduğu ancak larva döneminin ergin döneme göre daha duyarlı olduğu belirlendi. Çalışma, prodigiosin pigmentinin, *T. molitor*'a karşı kullanım için umut verici bir biyokontrol ajanı olabileceğini gösterdi.

Anahtar Kelimeler- Serratia Marcescens, Bakteriyal Pigment, Prodigiosin, Biyokontrol

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I. INTRODUCTION

Growers are trying to obtain maximum yield from limited cropland to meet the food needs of the world's growing population. On the other hand, they are struggling with pests that cause yield losses in agricultural production in order to achieve sustainable nutrition.

The yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is a important pest of stored products such as flours, starches and pastas [1]. It not only eats the product but also contaminates it with body fragments and faeces, leading to a decline in food quality [2]. Currently, the main approaches to controlling this pest are based on conventional chemical insecticides. However, their unconscious, overuse has led to the development of resistant populations, the elimination of their natural enemies, and the outbreak of secondary pests. These are limited their application. Therefore, an environmentally friendly alternative is needed to overcome these limitations and achieve high yield. Microorganisms and their products could be the best choice to replace the wide use of conventional chemical insecticides.

Serratia marcescens (Enterobacteriaceae: Serratia) is one of the most important microbial control agents, controlling a variety of economically important pests. The bacterium is commonly found in various insects such as *Antheraea pernyi* (Lepidoptera: Saturniidae) [3], *Phyllophaga blanchardi* (Coleoptera:Scarabaeidae) [4], *Spodoptera exigua* (Lepidoptera: Noctuidae) [5], *Curculio dieckmanni* (Coleoptera: Curculionidae) [6]. The primary metabolite chitinases plays an important role in the insecticidal activities of *S. marcescens* by hydrolyzing the body wall and peritrophic membrane structure of the insects [7]. On the other hand, *Serratia marcescens* produces a red pigment called prodigiosin as a secondary metabolite that has biological properties such as antibacterial [8], antifungal [9], antimalarial [10], nematicidal [11], immunosuppressive [12], and anticancer [13] properties. It also showed insecticidal activity on insects. Larvicidal effect on *Aedes aegypti* and *Anopheles stephensi* has been previously reported [14], [15]. In addition, insecticidal potential has been reported on adults of *Periplanata americana* [16], nymphs of *Diaphorina citri* [17] and larvae of *Helicoverpa armigera* and *Spodoptera litura* [18] Studies on the insecticidal potential of prodigiosin on insect pests were limited in these studies.

Thus, the objective of the present study was to extract a crude prodigiosin pigment from a native *Serratia marcescens* Se9 isolate and determine its insecticidal potential on the larvae and adults of *Tenebrio molitor*. This is also the first study on the efficacy of prodigiosin on *Tenebrio molitor*.

II. MATERIALS AND METHODS

A. Culture of Serratia marcescens

Serratia marcescens Se9, a red pigmented bacterium isolated from larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae) in a previous study, was used [5]. A loopful of bacterial suspension from a glycerol stock culture was spread onto nutrient agar plate and incubated at 30 °C for 24 hours. Then a single colony was transferred to a fresh nutrient agar. It was used for further studies after ensuring that the culture was pure.

B. Production and extraction of prodigiosin

A 24-h-old culture of *S. marcescens* Se9 was inoculated in 250 ml of nutrient broth medium and incubated for 48 h at 30 °C under static conditions. The pigmented culture was then centrifuged at 10,000 rpm for 15 minutes at 4°C, and the supernatant was discarded. Extraction of prodigiosin from the cells was carried out as described by Suryawanshi et al. [15] with a slight modification. The pellet was resuspended in acidified ethanol (4.0 mL 1 N HCI - 96.0 mL ethanol) and vortexed vigorously for 5 minutes. The suspension was centrifuged at 10,000 rpm for 15 minutes, and the supernatant containing the prodigiosin was carefully transferred to a sterile Falon tube (50 mL). This procedure was repeated until the pellet was colorless. The solvent was removed under vacuum in a rotary evaporator (Bibby Scientific Ltd, Staffordshire, UK) at 50 °C with chiller temperature set at below 10 °C until the dried red pigment was obtained. After the crude pigment was collected and quantified on a dry weight basis, it was stored at 4 °C until used for the bioassay.

C. Insect rearing

Tenebrio molitor used in the bioassay were obtained from a local pet shop. To confirm that the insect was *T. molitor*, larvae were examined under a stereomicroscope to reveal characteristic features such as the evenly divided linear grooves extending the entire length of the abdomen and four tarsal segments on the hind legs [19]. The larvae were kept in disenfected plastic boxes (50×30×10 cm) and maintained at 25 ± 1 ºC, 70 ± 5% relative

humidity (RH) with a light/dark 12:12 h photoperiod. The larvae were fed *ad libitum* with whole grain flour (90%) and instant dry yeast (10%). In addition, a piece of fresh-cut potato were placed in the boxes for providing the necessary water to insects [20].

D. Bioassays

Twenty mg of the dry pigment was resuspended in 10 ml of sterile 96% (v/v) ethanol and filtered through a 0.20 µm sterile syringe filter (Minisart, Sartorius, Germany). Five different concentrations of the pigment (2000, 1000, 500, 250, 125 ppm) were then prepared by 1:2 dilution and insecticidal activity was tested on both larvae (14th instar stage) and adults (newly hatched) of *Tenebrio molitor* by leaf disk feeding assay [21]. Disks 5 cm in diameter cut from cabbage leaves were dipped in the concentrations prepared from the pigment for 5 seconds and then allowed to dry for 30 minutes to evaporate the ethanol. The disks used in the control group were dipped into sterile ethanol. The treated and control disks were placed individually in Petri dishes. At each concentration, twenty 14th instar larvae were placed on a treated leaf disk in Petri dish. The experiments were performed triplicate for each concentration and control group. The experiment was conducted at 25 °C and 60% relative humidity with a light/dark 12:12 h photoperiod. Mortality was recorded daily for 5 days. Experiments were also performed with adults as described above.

E. Data analysis

Mortality data were corrected with Abbott's formula [22]. The Kaplan–Meier method was used to plot cumulative survival curves. Log-rank (Mantel-Cox) test was used to assess difference in survival between each concentrations. Probit regression analysis was performed to determine prodigiosin concentration required to kill 50% and 90% of the pest (LC_{50} and LC_{90}) was calculated. All the analyses were conducted using SPSS Statistics 25 software package (SPSS Inc., Chicago, IL, USA).

III. RESULTS

The pigment prodigiosin was extracted from *Serratia marcescens* Se9 grown in nutrient broth medium using acidified ethanol as solvent. After evaporation of the solvent by a rotary evaporator, the crude pigment was obtained. Different concentrations from 2000 to 125 ppm (1:2 dilution) were prepared from the crude pigment and tested on the larvae and adults of T. molitor. It was found that the mortality rate of larvae exposed to the lowest concentration (125 ppm) of the crude pigment was 5%, while the mortality rate of larvae exposed to the highest concentration (2000 ppm) was 68%. In addition, at a concentration of 1000 ppm, a mortality of 73% was observed. Log-rank analysis showed that the larvae of T. molitor exposed to 2000, 1000 and 500 ppm of pigment concentration were statistically different from the control group (Table 1). However, there were no significant differences in mortality between 250, 125 ppm and the control group.

Table 1. Log-Rank (Mantel–Cox) analysis result of *Tenebrio molitor* larvae exposed to different concentrations of crude prodigiosin pigment

Concentrations (ppm)	2000		1000		500		250		125		Control	
	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*
2000			0.003	0.959	43.197	0.000	83.953	0.000	87.411	0.000	96.919	0.000
1000	0.003	0.959			48.227	0.000	92.810	0.000	96.849	0.000	106.79	0.000
500	43.197	0.000	48.227	0.000			10.954	0.001	11.476	0.001	17.826	0.000
250	83.953	0.000	92.810	0.000	10.954	0.001			0.001	0.978	1.364	0.243
125	87.411	0.000	96.849	0.000	11.476	0.001	0.001	0.978			1.330	0.249
Control	96.919	0.000	106.799	.0000	17.826	0.000	1.364	0.243	1.330	0.249		

X^2: Chi-square, *p*: significance

Figure 1A illustrated the survival curves of the larvae in the control group and the infected group. The control group had the highest survival rate, while the larvae exposed to the pigment at a concentration of 1000 ppm had the lowest survival rate. The LC_{50} value of the crude pigment for the larval stage was determined to be 924 ppm (Table 3). On the other hand, even at the highest pigment concentration (2000 ppm), only 30% mortality was observed in adults. While there was a statistical difference in mortality between the 2000, 1000, 500 ppm concentrations and the control group, there was no difference in mortality between the 250, 125 ppm concentrations and the control group (Table 2). Figure 1B illustrated the survival curves of the control group and the infected adult groups. The control group had the highest survival rate, while adults exposed to the pigment at

a concentration of 2000 ppm had the lowest survival rate. The LC50 value of the crude pigment for the adult stage was determined to be 4573 ppm (Table 3).

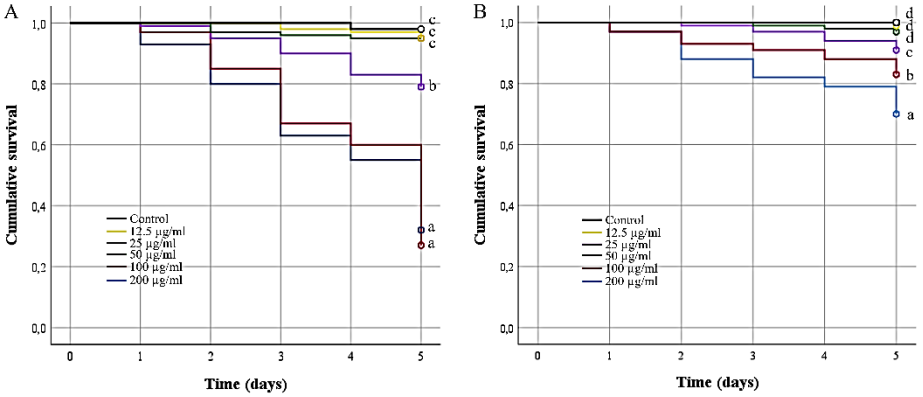

Figure 1. Kaplan-Meier survival diagram for larvae (A) and adult (B) of *Tenebrio molitor* exposed to crude prodigiosin pigment at five different concentrations. The lowercase letters represented the significant differences between concentrations.

Tablo 2. Log-Rank (Mantel–Cox) analysis result of *Tenebrio molitor* adults exposed to different concentrations of crude prodigiosin pigment

Concentrations (ppm)	2000		1000		500		250		125		Control	
	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*
2000			4.598	0.032	14.458	0.000	26.652	0.000	32.316	0.000	35.235	0.000
1000	4.598	0.032			2.960	0.085	10.953	0.001	15.702	0.000	18.503	0.000
500	14.458	0.000	2.960	0.085			3.182	0.074	6.763	0.009	9.378	0.002
250	26.652	0.000	10.953	0.001	3.182	0.074			1.028	0.311	3.030	0.082
125	32.316	0.000	15.702	0.000	6.763	0.009	1.028	0.311			1.000	0.317
Control	35.235	0.000	18.503	0.000	9.378	0.002	3.030	0.082	1.000	0.317		

X^2: Chi-square, *p*: significance

Table 3. Median lethal concentration (LC_{50}) of crude prodigiosin pigment on different life stages of *Tenebrio molitor*.

Life stage	LC_{50} (ppm)	95% *Cl*		Slope ± *SE*	LC_{90} (ppm)	*df*	X^2
		Lower bound	Upper bound				
Larvae	924	414	5738	2.14 ± 0.18	3494	3	28.1
Adult	4573	2946	9872	1.42 ± 0.22	34384	3	0.2

Cl: confidence limits, *SE*: standard error, *df*: degree of freedom, X^2: chi-square.

IV. DISCUSSION

The pathogenicity and virulence of various microorganisms and their products have been evaluated for biological control of *T. molitor*. Specially, the efficacy of entomopathogenic microorganisms *Beauveria bassiana*, *Metarhizium anisopliae* and *Bacillus thuringiensis* on different developmental stages of *T. molitor* has been reported [23]–[26]. However, there is no study on the efficacy of bacterial pigments against *T. molitor*.

In this study, the insecticidal activity of the prodigiosin pigment produced by a native *S. marcescens*, which is reported to have many biological properties, was tested for the first time on larvae and adults of *Tenebrio molitor*. The larval stage was more susceptible to the prodigiosin pigment than the adult stage. The LC_{50} value for the larval stage was about five times lower than for the adult stage. However, compared to previous studies, the LC_{50} values were high. Liang et al [27] tested the prodigiosin pigment extracted from *Serratia marcescens* TKU011 on *Drosophila melanogaster* larvae, and the lethal concentration that caused 50% larval mortality was reported to be 230 ppm. Similarly, the larvicidal effect of prodigiosin extracted from *S. marcescens* NMCC46 on mosquitoes revealed LC_{50} values of 103.95 and 105.52 ppm against third instar larvae of *A. aegypti* and *An. stephensi*, respectively [14]. In our study, the LC_{50} values for larval and adult stages of *T. molitor* were 924 and 4573 ppm, respectively. In contrast, Zhou et al [28] reported that the red pigment prodigiosin is not a major virulence factor for entomopathogenic *Serratia marcescens*. They tested the pathogenicity of pigmented *S.*

marcescens and its non-pigmented mutant strain against larvae of *Bombyx mori* and found that the LC_{50} values were similar, but the larvae that died from the pigmented bacteria turned red.

The difference in LC_{50} values between these studies can be explained by the immune response of the insects to prodigiosin. Inhibition of immune system enzymes such as protease phosphatase, acid phosphatase and acetylcholine esterase has been reported when prodigiosin was exposed to insects [15]. It also leads to a drop in pH in the insect's midgut, which can result in reduced nutrient uptake and lead to the insect's death. However, resistant insects can overcome this situation by a detoxification mechanism through high activity of esterases. Therefore, the susceptibility of insects to the pigment prodigiosin may vary.

Kaplan-Meier analysis showed that the survival rate of both larvae and adults in the control groups was similar to that of insects treated with 125 and 250 ppm prodigiosin (Tables 1 and 2). However, the survival rate of insects treated with the concentration of 500 ppm decreased significantly, reaching 31.7% in larvae and 70% in adults at the concentration of 2000 µg/ml. A similar trend was reported by Patil et al. [14] that increased concentration of the pigment prodigiosin resulted in lower survival rate in larvae of *A. aegypti* and *A. stephensi*. Furthermore, at a concentration of 500 ppm, they observed complete mortality on the larvae of *A. aegypti* within 48 hours. In contrast, the survival rate of *T. molitor* larvae at 200 µg/ml concentration was 31.7% within 5 days.

V. CONCLUSION

The study exhibited that the prodigiosin pigment produced by *S. marcescens* has insecticidal potential for *Tenebrio molitor*. The larvae of the insect are more susceptible to prodigiosin pigment than the adult stage. The pigment appears to be a promising biological control agent for the integrated menegement of *T. molitor* larvae. It can be used alone or together with other biological control agents. However, the synergistic effect with other biological control agents should be determined.

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