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EXTRACTION OF THE RIBOSOME-INACTIVATING PROTEINS FROM THE LEAVES OF THE *DIANTHUS CARYOPHYLLUS* L. AND EVALUATING ITS EFFECT ON SOME ASPECTS OF THE BIOLOGY OF *TROGODERMA GRANARIUM* EVERTS (COLEOPTERA: DERMESTIDAE)

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Abstract

The current study was conducted to extract the Ribosome-inactivating proteins from the leaves of the *Dianthus caryophyllus* L. and assessing the content of amino acids in it, in addition to evaluating its effect on some aspects of biology of *Trogoderma granarium* Everts when mixed with grains and wheat flour and rearing the insect on it using three concentrations 0.025, 0.05, 0.1 µg for the period from August until October in the Mosul city, Iraq. The study demonstrated presence of the RIPs in the leaves of the carnation plant and that the extracted amount from 25 grams of leaves was 175.3379 µg, the study also indicated that they contain all the amino acids except the leucine and lysine using HPLC technique. Furthermore, the results showed that all the concentrations resulted in the elongated of the larval, pupal and adult stages then increasing generation and generation time compared with the control treatment and average duration of the insect generation time was 48.7, 45.1 and 56.4 days at the concentrations 0.025, 0.05, 0.1 µg, respectively, while it was in control treatment 32.3 days.

Key words : *Trogoderma granarium,* Ribosome-inactivating proteins*, Dianthus caryophyllus.*

Introduction

The *Trogoderma granarium* Everts beetle is one of the most important pests of grains and major stored products in most countries of the world, especially tropical and subtropical countries. It is also found in countries moderate temperate where the temperature ranges between 32-44 ° C for a period of no less than four months of the year and in Iraq, it is considered a very dangerous pest, especially for grains and their products (Al-iraqi, 2010). The rate of development and generations time of the Khabra beetle varies depending on the type of host, temperature, light, moisture content, and season. Depending on these factors, the insect may have 1-9 generations per year. Adults live from 12 to 25 days and the female lays about 50-100 eggs. The larval stage is 4-6 weeks, and the larvae molt between 4-15 times. The pupal stage is 2-5 days, However the larval stage may last for months and years if the larva enters diapauses. The larva has the ability to survive for several years without food (Szito, 2007). Through its long history in its struggle with insects that infect grains and stored products, man has used many natural plant materials available in his environment in various ways to reduce the harm of these pests, Attention, investigation and detection of many plant materials has increased in recent decades in various countries of the world for use as alternatives to chemical pesticides to control these pests, One of these alternatives which is possible used is Ribosome-inactivating proteins (RIPs) who is considered a toxic N-glycosidases that depurinate eukaryotic and prokaryotic rRNAs, thereby arresting protein synthesis during translation and widely

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found in various plant species within different tissues. It is demonstrated in vitro and in transgenic plants that RIPs have been connected to defense by antifungal, antibacterial, antiviral, and insecticidal activities (Zhu *et al.,* 2018). So this study was conducted to assess the effect RIPs that extract from the leaves of the carnation plant on the duration of the larval, pupal, adult stages, generation and generation time of Khabra beetle.

Materials and Methods

1. The plant used in the study

Danthus caryophyllus L., Hybrid nana variety (Pink color) was used in this study provided by Palm Paradise Company for Textile Agriculture Ltd. in Baghdad / Iraq.

Protein Extraction

Plant Extraction Buffer

The extraction buffer was prepared according to the method described by (Park *et al.,* 2006). To prepare 1 liter of the extract solution we need: -

1. 25 Mm phosphate buffer PH 7.0
2. 250 Mm NaCl
3. 10 Mm Ethylene Diaminetetraacetic acid (EDTA)
4. 5 Mm Dithiothreitol (DTT)
5. 1 Mm phenylmethyl Sulfonyl Fluoride (PMS F)
6. 1.5% [W/V] Polyvinylpoypyrrolidone (PVP)

RIPs Extraction

Extraction process was performed according to the following steps:

1. Plant leaves are ground in liquid nitrogen along with acid-washed sand for maximum cell disruption.
2. 6 ml of extraction buffer solution is added to the crushed sample and placed in a centrifuge at a speed of 10,000 r / min^{-1} for 30 minutes.
3. The supernatant is transfer into a clean glass and 30 ml from ammonium sulphate is added to a final concentration of 20% (w/v) with continuous stirring to avoid agglomeration of ammonium sulphate.
4. The mixture is placed in the refrigerator for an hour, after which it is placed in a centrifuge for 30 minutes at a speed of 10,000 r / min^{-1}.
5. We add 30 ml of ammonium sulfate gradually to the cooled solution and very slowly taking into account that the foam did not form during addition until the calculated amount is completed.
6. Centrifugation works at 10,000 r/ min -1 for 30 min for samples and then we take precipitate and discard leachate.
7. Dialysis works for the sample and the dialysis tube is left for 24 hours, as the solution produced after dialysis is considered the extracted protein.
8. All extraction procedures are conducted at 4°C, and the ammonium sulphate fraction is stored at –80°C until use (Park *et al.,* 2006).

Estimate the protein concentration

The method described by Bradford and Heath (1976) was used to estimate protein concentration using the standard curve for Bovine Serum Albumin (BSA) as in (Fig. 1).

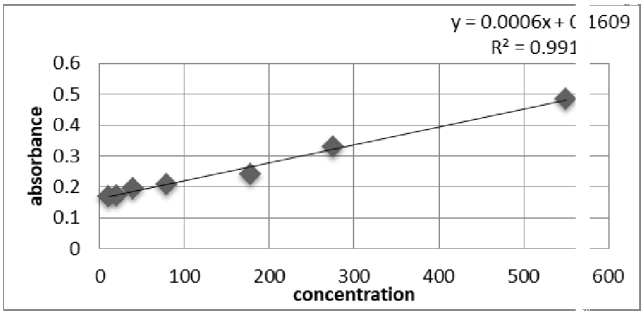

Fig. 1: The Standard Curve for Bovine Serum Albumin (BSA).

Estimating the amino acid content in the protein

Extraction and Separation of amino acids

A weight of 1 mg was taken from the study samples, and mixed with 50 ml ethanol: water with a volumetric ratio 60: 40 respectively, then digest the sample extract in 30 ml of HCl acid (6N), The sample was transferred to glass tubes and left in a dry incubator at 110 ° C for 6 hours. The decomposed sample was filtered using vitreous filters, then concentrate the filtrate to 5 ml with nitrogen gas vapor. After the concentration, the sample was mixed with 5 ml of acetic acid (0.1 M) with PH 2.2. Finally, 10 ml of Phenyl isothiocyanate (PITC) was added, at which point the sample was ready for injection into the HPLC machine (Cohen *et al.,* 1986).

Quantitative diagnosis and Estimation of amino acids using HPLC Technique

The separation and diagnosis process of the amino acids extracted from the samples (Paragraph 4-1) was performed using a High- performance Liquid Chromatography technique (HPLC) due to the high efficiency and accuracy of this technique in carrying out a quantitative and qualitative assessment of the compounds to be diagnosed from the calculation of the curves and their height and determining the concentration of this

compound in one process (Furst *et al.,* 1990). Use a Japanese-made HPLC (UFLC SHMADZU) using the metallic separation column with specifications:

Properties	Specifications of amino acid separation column
Stationary phase	XR-ODS
Column length	50 mm
Inner diameter of the column	4.6 mm
The diameter of the filling granules	3 Microliter

Conditions for diagnosing the standard form of amino acids and compounds separated from the selected samples:

Conditions	Amino acids
Mobile phase (v:v)	40 water :60 ethanol
Mobile phase flow speed	1 ml / minute
The volume of the injected sample	20 Microliter
Separation temperature	110 ° C
Detector type (Furst *et al.*, 1990)	Ultraviolet radiation (UV) at a wave length of 254 nanometers

The diagnostic process for the samples under study was carried out based on the standard model of amino acids (Fig. 2). Tests were performed in the Ministry of Science and Technology Laboratory-Baghdad.

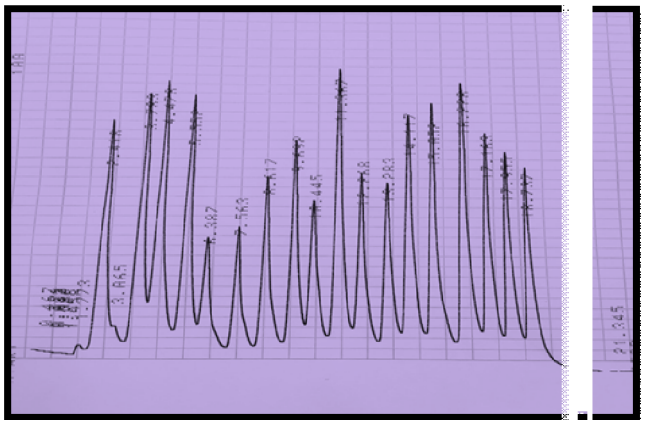

Fig. 2: Curves for standard sample amino acids.

The appropriate readings are taken, including the area of the curve and the retention time, through which the isolated compound can be diagnosed from the samples compared to the standard sample calculation time, and the percentage ratio is calculated according to the following equation (Okamura and Fujimote, 1994).

Concentration: Curved area for sample ÷ Curved area for standard × Concentration × Number of dilution times

Compound percentage : T/ S × 100

Where T: represents the area of the curve of the compound isolated from the tested samples.

S: represents the curve area of the standard compound.

The insect under study

In this study, *Trogoderma granarium* Everts (1898) Coleoptera: Dermestidae was used taken from farms breeder for several years in the Insect Research Laboratory in the Biology Department, College of Science, University of Mosul.

Rearing of insects

The insect was raised on whole, clean, wheat grains (variety EBAA) taken from the Seed Inspection and Certification Service / Nineveh, as it was manually cleaned to remove foreign materials and sifted them, then put in electric oven (60 ± 5 C°) for eight hours to remove any possible infection (Sharma, 2013) and it was placed in the incubator at a temperature 32-35 ° C and relative humidity 55 ±10 in glass bottles (15.5 cm x 5 cm), the number of last-instar larvae and adult insects (male and female) of the Khabra beetle added to them and covered with muslin fastened with elastic bands which were considered the mother farm (Hou and Fields, 2003a).

Effect of RIPs on some aspects of the Biology of Trogoderma granarium

A number of pupa taken from breeding farms were isolated and placed in a Petri dish and left in the incubator under daily control until the adults emerged from them. A number of freshly emerged adults (male and female) were taken, and transferred to a Petri dish containing wheat flour as food and left in the incubator until the eggs were laid, the eggs were monitored until hatching and the first larval instar emerging, then ten first instar larvae (a few hours of age) was kept individually in separate 10 glass vials containing 1 gram of whole and broken wheat grains (per vial) and treated with extracted protein and at concentrations 0.025, 0.05, 0.1 µg (one larva per vial), the vial nozzle was covered with a small piece of cotton, and placed in incubator at a temperature of 32-35 °C and relative humidity 55 ±10. The development of the larvae was monitored daily until it reached to adults. Means of larval, pupal, adult durations, generation and generation time was recorded. The experiment was performed at 10 larvae for each concentration in addition to the control treatment (Ishimoto and Kitamura, 1988; Ahmedani *et al.,* 2009; Yadav *et al.,* 2018).

Results and Discussion

Ribosome-inactivating proteins extraction

The results demonstrated in Fig. 3 the presence of

the RIPs in the leaves of the carnation plant and that the extracted amount from 25 grams of leaves amounted to 175.3379 µg, These results are consistent with (Stirpe *et al.,* 1981) Which is the first to isolated and describe RIPs in leaves of *D. caryophyllus* where they isolated two types of protein are dianthin-30 and dianthin-32.

Amino acids present in RIPs and its percentage

The curves recorded from the injection of a sample of RIPs extracted from the carnation plant in the HPLC indicated that they contain all the amino acids except the Leucine and Lysine, The percentage of amino acids present in the protein RIPs (Table 1) differed to record the highest value of 11.46% in the amino acid Alanine and the lowest of 0.62% in the amino acid Glutamic.

Table 1: Amino acids present in RIPs and its percentage.

Amino acids	Concentrations (µg)	Percentage
Aspartic acid	834,54	10,13
Glutamic acid	51,68	0.62
Serine	494,74	6.00
Arginine	305,73	3.71
Aspargine	566,55	6.88
Cysteine	888,29	10.78
Alanine	944,06	11.46
Proline	488,34	5.93
Glycine	424,59	5.15
Threonine	374,49	4.54
Tyrosine	313,47	3.80
Valine	466,29	5.66
Methionine	79,30	8.61
Histidine	668,29	8.11
Isoleucine	226.78	2.75
Leucine	-	-
Phenylalanine	475,77	5.77
Lysine	-	-
Total	7602,91	

Effect of RIPs on some aspects of the Biology of Trogoderma granarium

Generally, the results in table 2 show that mixing the of RIPs with wheat grains and rearing the insect on its has elongated the duration of larval, Pupal, Adult stages, Generation and Generation Time than in the control treatment and the longest duration of the larval stage reached to 34.2 day at the concentration of 0.1 µg, while the shortest duration of the larval stage 26.7 day at concentration 0.05 µg, while in the control treatment it was 19 day. Also, the results showed that the duration of the pupal stage ranged in length from 6.2 days at concentration 0.025 µg and 5 days as the shortest period recorded at concentration 0.05 µg, as for the control treatment, it reached 5.4 days. Moreover, With regard to the duration of generation, which is the period from eggs hatching until the adult emerge; the highest duration of generation was 40.2 days at concentration 0.1 µg, while the lowest duration of generation 31.7 days was at concentration 0.05 µg. On the other hand, the results show that there is a variation in the duration of adult stage and the adult stage showed its longest lifespan in the concentration state 0.1 µg it reached 16.2 days, while its shortest lifespan in the concentration state 0.05 µg it reached 13.4 days. From the above, It is clear that the longest generation time reached 56.4 days at the concentration 0.1 µg, while the lowest generation time was 45.1 days at concentration 0.05 µg, from the above we also conclude that all concentrations elongated the generation time compared to the control treatment that was 32.3 days. Several studies have demonstrated that the leaves of the carnation plant contain RIPs, which is Dianthin 30/32 (Stirpe *et al.,* 1981; Park *et al.,* 2006) and it is a toxic protein possess insecticidal activity upon different insects such as Lepidoptera (Wei *et al.,* 2004), Coleoptera (Kumar *et al.,* 1993) and Diptera (Shahidi-Noghabi *et al.,* 2008) and the latest study also found that the feeding of *Myzus nicotianae* with leaves from

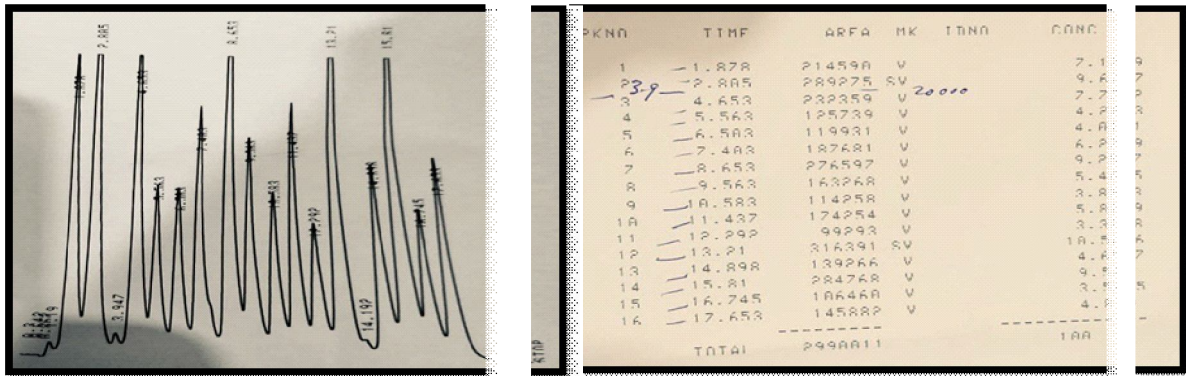

Fig. 3: Curves for Amino acids present in RIPs.

Table 2: Effect of RIPs on some aspects of the biology of *Trogoderma granarium.*

Concentrations (µg)	Mean (days) ± SE				
	Duration of larval stage	Duration of Pupal stage	Generation	Duration of Adult stage	Generation Time
0.025	28.1± 1.64	6.2±0.66	34.3±1.59	14.4±1.59	48.7±0.91
0.05	26.7± 0.94	5±0.29	31.7± 0.88	13.4± 2.73	45.1± 3.45
0.1	34.2±2.43	6.0± 0.49	40.2± 2.6	16.2±2.41	56.4± 4.28
Control	19.2± 0.57	5.4±0.6	24.6± 0.71	7.7± 0.57	32.3±0.57

transgenic tobacco plants overexpressing SNA-I retarded the generation time and decreased the fecundity and adult survival, From the above we conclude that giving RIPs with the insect's food may be the reason for its negative impact on the generation time and the different stages of the insect.

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