Evaluation of the efficacy of extracts of two types of Mollusca to control the larvae of the greater wax moth Galleria mellonella (lepidoptera pyralidae) by contact

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Abstract
Extracts of two types of molluscs were used, one of which is the ethyl alcohol extract of the soft tissues of the oyster *Psudontopsis euphraticus* (Burginat, 1852) and the aqueous extracts of its shells and their mixture under concentrations (10, 20, 30 and 40) mg.ml^{-1}, with the GC-MS and X-ray fluorescence technology for extracts. The other one is a commercial preparation which is a protease enzyme of *Achatina fulica* snail under concentrations (2.5, 5, 7.5 and 10) mg.ml^{-1}, to find out the effect of the two species in controlling the larvae of *Galleria mellonella* (L.) and to compare the results using a new insecticide to control insects of the Lepidoptera known as Belt$_{480SC}$ (Flubendiamide) at concentrations (0.1, 0.2, 0.3 and 0.4) ml.L^{-1}.
The results recorded a mortality rate of (100)% for the first instar at all concentrations, while the death rates differed for 4th and 7th instars. In the treatment of the 4th instar, soft-tissue extract exceeded the mortality rate of (100)% at a concentration of 40 mg.ml^{-1}, then the Belt$_{480SC}$ and mixture treatments with close values (86.66, 76.66)% respectively, while the shell extract and protease treatments ranked third with close values (36.66 and 43.33)% respectively after 120 hours at the highest concentrations, while the 7th instar, it was noticed that when the two treatments with (soft-tissue extract and Belt$_{480SC}$) were equivalent to (70.00)%, while treatment with the mixture and protease ranked second with the effect of (43.33 and 26.66)% respectively, While the treatment with shell extract decreased by (10.00)%.
Keywords: Galleria mellonella, Protease, Mollusca, Larvae, Flubendiamide.

1. Introduction

The great wax moth *mellonella Galleria* (L.)belonging to the family Pyralidae is one of the most important pests that attack wax frames in beehives or in dark warehouses in particular and cause the death of large numbers of bee colonies annually in many places of their breeding, and cause severe economic damage (17; 23; 27).
In this study, Belt$_{480SC}$, which is one of the modern insecticides belonging to the new chemical group Benzene Dicarboxamide or Phthalic Acid Diamide, discovered by Nihon Nohyaku and developed jointly with Bayer Crop Science, was used. The chemical group is characterized by the strength and speed of effectiveness against a wide range of economically important lepidoptera pests, including resistant strains, containing 480 g.l^{-1} of the active substance flubendiamide, and its formula is in the form of a water-suspension concentrate or white crystalline powder. By contact and ingestion, highly permeable (penetrating), lipophilic in nature, broad spectrum against all stages of larvae of the order Lepidoptera (14).
Also, extracts of two species of Mollusca were used: *P. euphraticus*, which belongs to the class Bivalvia, and the species *A. fulica*, which belongs to the class Gastropoda. The benefit of these invertebrates comes from the presence of a group of active components in the form of carbohydrates, proteins, minerals, fats, sterols and nucleosides (12).
Numerous studies have demonstrated the effectiveness of extracts of various types of molluscs in combating various types of bacteria and fungi (1; 4; 13). From here, the study included the use of extracts of two types of molluscs (oysters and snails) in the control of larvae of the greater wax moth insect to demonstrate the efficiency of using such animal extracts, similar to plant extracts, as one of the vital, safe and alternative means to chemical pesticides.

2. Materials and Methods

Preparation of extracts:- 1

1-1 Alcoholic extract of the soft tissues of oyster

The outer shell was opened, the soft part was extracted, washed with tap water, then with sterile distilled water, then dried with cellulose paper. These parts were placed with an equivalent of 70% ethyl alcohol (every 100 g / 400 ml) in an electric blender and mixed for 3 minutes until the solution was homogeneous. Leave the mixture for 24 hours on a magnetic stirrer at a temperature of (4°C), the mixture was then centrifuged at 4000 r/m for 3 minutes at room temperature (4), The liquid was poured into glassware (Pyrex) containers, and then placed in an incubator at a temperature of (45°C). After drying, the sample was scraped, placed in sterile glass vials, labeled,

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and kept in a refrigerator until use.
The stock solution of the alcoholic extract was prepared by dissolving 4 g of dry residuals in 5 ml of 70% ethyl alcohol and mixing well with a magnetic mixer for 10 minutes, then the volume was added to 100 ml of distilled water to obtain a 4% solution, which is equivalent to 40 $mg.ml^{-1}$ from it, the required concentrations were prepared in the experiment (10, 20, 30, and 40) $mg.ml^{-1}$, and two drops of Tween 20 were added to each concentration as a dispersant per 100 ml of concentrations, while the control was distilled water (30; 7).

1-2 Aqueous extract of oyster shells

To remove surface organic contaminants and the fleshy residue stuck in them, the shell is soaked in a brine solution (saturated with sea salt) for 24 hours, after which it is washed thoroughly with tap water, then with sterile distilled water, and then dried in the shade for several hours. The shells were ground using an electric mill several times, and then the resulting powder was sieved using a Chinese standard sieve with 0.25 mm holes (5 , 29)
The aqueous extract was prepared according to the method of 3 and 15 with some modifications. Soak 100 g of shell powder in 500 ml of distilled water for 12 hours at room temperature with continuous stirring by a magnetic mixer, and filter the extract using Whatman filter papers. No. 1, then the aqueous extract was dried using an electric oven at a temperature of (45 °C), then placed in sterile glass containers and marked in the refrigerator until use.
The stock solution of the aqueous extract was prepared by dissolving 4 g of dry residual in 100 ml of distilled water and mixing well to obtain a 4% concentration solution, which is equivalent to 40 $mg.ml^{-1}$, and from it the required concentrations were prepared in the experiment (10, 20, 30 and 40) $mg.ml^{-1}$, and two drops of Tween 20 were added to each concentration as a dispersant for every 100 ml of concentrations, while the control treatment was distilled water (30; 7).

1-3 Commercial preparation of protease enzyme to the snail A. fulica

A commercial product in the form of a fine, milky powder extracted from the snail of *A. fulica*, known as the Chinese white jade snail, the pH ranges from (6.1-6.5), stored in a cool, dry place away from light at 2-8 °C. Highly soluble in water, produced by Yangling Ciyuan Biotech Co., LTD located in Xi'an City, Shaanxi Province, China, and was imported by alibaba marketing company to Iraq.

2 - Chemical detection of active substances in the tissues and shells of oyster

2-1 Gas Chromatography Mass Spectrum (GC-MS) Analysis

The main compounds of the alcoholic tissues extract of *P. euphraticus* were determined by GC-MS, and the analysis was carried out at the Ministry of Science and Technology/Department of Environment and Water/Environmental Research Center.

The main compounds of aqueous extract of oyster shells of *P. euphraticus* were identified by GC-MS, and the analysis was carried out at the Industrial Research and Development Authority.

2-2 X-ray fluorescence (XRF) analysis

The weight of 6 grams of the aqueous extract of the shells was taken, then the binder boric acid was added to it, and after homogeneous mixing, the mixture was pressed into discs with a diameter of (40) mm and a thickness of (5) mm. Then the discs were inserted into an X-ray fluorescence (XRF) type S8-TIGER to determine the metals and oxides in them. The analysis was carried out in the Ministry of Science and Technology / Department of Environment and Water / Environmental Research Center.

3- The different transactions used in the study

The executed transactions included the following:
-First treatment: Belt480sc with concentrations of (0, 0.1, 0.2, 0.3 and 0.4) $ml.L^{-1}$.
-The second treatment: an alcoholic extract of the soft tissues of oysters at concentrations of (0, 0, 1, 20, 30, 40) $mg.ml^{-1}$.
-Third treatment: an aqueous extract of oyster shells at concentrations of (0, 10, 20, 03, 40) $mg.ml^{-1}$.
-The fourth treatment: Mixing the alcoholic extract of soft tissues with the aqueous extract of the peels at the same concentrations above.
-Fifth treatment: Snail protease enzyme preparation at concentrations of (0, 2.5, 5, 7.5 and 10) $mg.ml^{-1}$.

4- Effect of different treatments on larval stages (first, fourth and seventh) of the greater wax worm.

The three larval instars (first, fourth, and seventh) of the wax worm were prepared from the pure colony, and three replicates were used for each instar and for each concentration separately at a rate of 10 larvae for each replicate. For the first larval instar, petridishes with a diameter of 9 cm were provided, and for the fourth and seventh instar, glass bottles with a diameter of 8 cm and a height of 15 cm were prepared.
The larvae of the three instar were placed in sterilized dishes with 30 larvae per plate, to carry out the treatments with the previously prepared concentrations by direct spraying with a small hand sprayer allocated for each treatment with a volume of 100 ml and from a distance of 15 cm and vertically for each treatment. The larvae were completely covered with the solution, as the portion of each dish was 3 ml for each treatment. Then, the treated larvae of the first instar were transferred with a soft brush to three petridishes 12 cm in diameter and 2.5 cm in height containing pieces of sterile wax for feeding, while the fourth and seventh instar larvae were transferred to three sterilized bottles. With a diameter of 8 cm and a height of 15 cm and also provided with sterile wax pieces, the mouths of the bottles were covered with muslin cloth and then tied with a rubber band, with a fourth repeat of the control treatment. The experiment was carried out under normal laboratory conditions, except for

protease treatment, which was carried out in the incubator at 25 ± 2 °C and 12 light hours.

5- Statistical design and analysis

Statistical Analysis System -SAS (36) was used to analyze the data to study the effect of different factors on the studied traits according to a factorial experiment that was applied in a completely randomized design-CRD, and the significant differences between the averages were compared with the Least Significant Difference test (LSD).

3. Results and discussion

GC-MS analyzes of ethyl alcohol extract of soft tissues and aqueous extracts of oyster shell *P. euphraticus* showed that the alcoholic extract contained approximately 26 active compounds distributed in groups such as glycosides, alkaloids, terpenes and esters in the soft tissues, and aqueous extracts of shellfish containing 15 compounds. Alkaloids, phenols, esters and alcohols, in addition to the presence of 13 metals and the presence of 14 oxides that increase their effectiveness as insecticides.

The results in Tables (1, 2 and 3) showed the effect of different treatments on the percentage of death of the larval instars (first, fourth and seventh) of the contact-treated great wax moth, as it was found that all the treatments used except for distilled water achieved a complete death rate of 100% for the first larval instar in all Concentrations with non-significant differences between them, while in the fourth larval stage, the alcoholic extract of the soft tissues of oyster maintained a high mortality rate that reached 100% compared to the rest of the treatments, as it was significantly superior to it. Likewise, in the seventh larval instar, the alcoholic extract of the soft tissues was the best, but with less efficiency from the previous stages, where the mortality rate reached 70% with non-significant differences with the pesticide, and the efficiency of each of the aqueous extract of shells and protease decreased on the fourth larval instar by 36.66 and 43.33% and on the seventh instar by 10.00 and 26.66%, respectively, compared to their effect on the first larval instar, which the mortality rate for them was 100%.

The current results also showed a high efficiency of 100% for all concentrations used on the first larval instar, then the effect of these concentrations decreased on the fourth and seventh instars, noting that the effect increases with increasing concentration in these two instars with significant differences, while distilled water had no significant effect.

Tables (1) show the effect of different treatments on the first larval instar and achieving a death rate of 100% for all contact treatments during different time periods ranging from (24, 72 and 120) hours and The relationship was inverse between the concentrations and the time needed to cause killing, as the extracts had the fastest effect within 24 hours, then the pesticide Belt$_{480sc}$, followed by the protease enzyme, which took 24 hours to 72 hours to cause killing compared to distilled water, in which the killing rate was zero.%

The reason for achieving these high rates of killing in the larvae of the first instar is attributed to the sensitivity of the instar because the body tissues in the first larval instar are fragile in addition to the incomplete defense system on one hand, and on the other hand the effectiveness of the compounds present in the pesticide, extracts and enzymes. This is consistent with what Keho (26) indicated when he stated that the early larval instars are more sensitive to the action of pathogens and extracts compared to the advanced larval instars represented by the third instar upwards, as the sensitivity of the larvae decreases with age, and the reason for this may be due to the rigidity of the body wall of the advanced larvae In old age, while newly hatched larvae, their body wall is less solid due to the decrease in the thickness of the cuticle layer and the ease of penetration of toxic compounds present in the extracts into the larva's body.

* The studied concentrations (except control = 0%) for the used treatments were as follows: for the pesticide: 0.1, 0.2, 0.3, and 0.4 ml.L^{-1}, for the extracts: 10, 20, 30, and 40 mg.ml^{-1}, and for the protease enzyme: 2.5, 5, 7.5, and 10 mg.mL^{-1}, respectively.

Accordingly, the first concentration, **Level I**, for the treatments, in order, is (0.1 ml.L^{-1}, 10 mg.ml^{-1}, and 2.5 mg.ml^{-1}), the second concentration, **Level II**, is (0.2 ml.L^{-1}, 20 mg.ml^{-1}, and 5 mg.ml^{-1}), and the third concentration, **Level II**, is (0.3 ml.L^{-1}, 30 mg.mL^{-1}, and 7.5 mg.mL^{-1}) and the **Level IV** concentration is (0.4 ml.L^{-1}, 40 mg.mL^{-1}, and 10 mg.mL^{-1}).

Table (1): The contact effect of concentrations of Belt$_{480SC}$, alcoholic soft tissue extract, aqueous extract of shells and their mixture of oysters *P. euphraticus* and protease enzyme of snail *A. fulica* on the mortality of first instar larvae of *G. mellonella*.

Concentrat-ions	Percentages of death of forth instar larvae					Concentr-ation rate
	Pesticide Belt 480SC	alcoholic extract of soft tissue	Aqueous extract of shells	mixture	protease enzyme	
Control	0.00	0.00	0.00	0.00	0.00	0.00
I	43.33	66.66	0.00	43.33	13.33	33.33
II	56.66	83.33	16.66	56.66	23.33	47.33
III	76.66	93.33	26.66	63.33	33.33	58.67
IV	86.66	100	36.66	76.66	43.33	68.67
Treatments rate	52.67	68.67	16.00	48.00	22.67	---
LSD 0.05	Treatm =* 9.37		Concen =* 9.37		16.83=* Overlap	

Table (2): The contact effect of concentrations of Belt$_{480SC}$, alcoholic soft tissue extract, aqueous extract of shells and their mixture of oysters *P. euphraticus* and protease enzyme of snail *A. fulica* on the mortality of forth instar larvae of *G. mellonella*.

Concentrat-ions	Percentages of death of first instar larvae					Concentr-ation rate
	pesticide Belt $_{480SC}$	alcoholic extract of soft tissue	Aqueous extract of shells	mixture	protease enzyme	
Control	0.00	0.00	0.00	0.00	0.00	0.00
I	100	100	100	100	100	100
II	100	100	100	100	100	100
III	100	100	100	100	100	100
IV	100	100	100	100	100	100
Treatments rate	80	80	80	80	80	---
LSD 0.05	Treatm =0.50 NS		Concen =0.50 *		1.0 NS= Overlap	

Table (3): The contact effect of concentrations of Belt$_{480SC}$, alcoholic soft tissue extract, aqueous extract of shells and their mixture of oysters *P. euphraticus* and protease enzyme of snail *A. fulica* on the mortality of seventh instar larvae of *G. mellonella*.

Concentrat-ions	Percentages of death of seventh instar larvae					Concentr-ation rate
	Pesticide Belt $_{480SC}$	alcoholic extract of soft tissue	Aqueous extract of shells	mixture	protease enzyme	
Control	0.00	0.00	0.00	0.00	0.00	0.00
I	26.66	30.00	0.00	10.00	0.00	13.33
II	40.00	43.33	3.33	26.66	10.00	24.67
III	56.66	53.33	6.66	36.33	16.66	33.92
IV	70.00	70.00	10.00	43.33	26.66	44.00
Treatments rate	38.67	39.33	4.00	23.27	10.67	---
LSD 0.05	Treatm =* 7.55		Concen =* 7.55		13.61=* Overlap	

As well as the effectiveness of compounds present in alcoholic and aqueous extracts of oysters, which may work together to affect the nervous system because they contain types of glycosides, alkaloids and terpenes according to GC-MS and minerals and oxides analyzes, and this is consistent with many studies that found that a large number of compounds present in extracts, they act in combination with each other, leading to changes that either prevent egg laying, affect the shedding hormone, or inhibit feeding (33). This study also agrees with the results of (8), where the first larval instar was the most sensitive to infection with some pathogens, and this was explained by the thinness of the cuticle compared to the third and fourth instar larvae, which have a thickened cuticle, which makes the first instar larvae more susceptible to infection.

Tables (2 and 3) showed a significant effect of the different treatments on the fourth and seventh larval instars, respectively, with different mortality rates when treated in contact during the time period (24, 72, and 120 hours). The alcoholic extract of the soft tissues of oysters has its high ability to cause complete paralysis of the larvae during the first minute of the first instar, and a period of time ranging from 1-1.5 minutes for the fourth and seventh instars, and the relationship was inverse between time and concentration to cause the effect, and the killing rate was high and reached (100% and 70 %) for the fourth and seventh instar, respectively, and this is attributed to the fact that the alcoholic extract contains a proportion of alkaloids known for their various toxicities, but they often involve neurotoxicity or disturbance of cellular signals (31, 32) compared to the aqueous extract of shellfish, which It took a longer time, exceeding two minutes, to cause paralysis of the advanced age larvae, because it contains one alkaloid represented by (1H,3H-Pyrrolo[1,2-c][1,3,2]oxazaborole, tetrahydro-1-methyl-3,3-diphenyl-), which is the most effective among the compounds of the aqueous extract of shellfish, according to the GC-MS analysis.

When comparing the compounds of extracts in the current study with plant extracts, we find that the extracts of many plant species such as oleander contain some elements in high quantities such as arsenine, silver, cadmium, chromium, copper, zinc and nickel (38). Moustafa *et al.* (33) found a relationship Between the content of the plant extract of these elements and its toxic effects on the insect *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae). This is consistent with the current results, as the gas chromatography analysis showed the presence of 13 metals, and calcium had the highest concentration 14.149%, while small amounts of chlorine 0.338%, sodium 0.169%, sulfur 0.140%, and a few parts per million of chromium, manganese and bromine were found. This is consistent with many findings that found the role of minerals in some extracts in

enhancing their toxic effect against insects (38; 42; 10; 28).

The effect of alcoholic extracts increases with increasing their concentration because, as was found in the GC-MS analysis, they contain many chemical groups that have an effect of inhibiting nutrition, such as mineral (inorganic) salts such as sodium, calcium, potassium, copper, and these substances, if found in high concentrations, are not accepted by the insect (9) and also extracts containing sulfur and some acids and bases as well as aromatic hydrocarbons, alcohols, aldehydes, ketones, non-protein amino acids, curmarines, alkaloids, sterols and terpenes. At high concentrations, the inhibitory effect of a substance is rarely general for all insects, and so far it is difficult to attribute these effects to a single chemical, or for the same substance to have a general effect on all insects (20).

The current results also showed the effect of the pesticide used Belt480sc (Flubendiamide) on the larvae in contact, as the percentage of killing the larvae in the first, fourth and seventh instars was 100, 86.66, and 70.00%, respectively, and this is consistent with the results of (39), which found a high efficiency of the pesticide. Flubendiamide in the control of lepidoptera insects. It affects the larvae with contraction symptoms that distinguish it from other insecticides, and it is very safe for non-target organisms. However, in the recent period (2) it was found that the extensive use of this pesticide has bad effects on the quality of products and human health, although it is one of the most commonly used pesticides on Lepidoptera.

Bayer Crop Science, (11) also indicated that the pesticide Belt480sc works by contact and ingestion and has a high permeability (penetration), with a lipophilic nature, and a broad spectrum against all stages of the larvae of the order Lepidoptera.

The reason for the superiority of the pesticide $Belt_{480SC}$ in causing killing of larvae is due to its specialization on ryanodine receptors (which are cellular calcium channels specialized in the intermittent and controlled transport of dense bursts of calcium ions that in turn cause muscle contraction), as the active substance flubendiamide (which is the first synthetic ryanodine receptor that has been developed marketed as insecticides) to interfere rapidly with ryanodin receptors located on the sarcoplasmic reticulum of muscles and the endoplasmic reticulum of neurons, epithelial cells and many other types of cells and loss of control over the liberation and transmission of calcium ions, which leads to the larva stopping feeding immediately with a sequential contraction of the larva's body followed by Complete non-retractable muscle contraction and consequent death of the lesion (24; 22; 21,40; 41).

The current results showed that the effect of protease enzyme by contact was stronger on the first larval instar compared to the advanced instar (fourth and seventh). This is explained by the small size of the first larval instar and thinness of the body wall in it due to the weakness of the thickness of the chitin layer, in addition to the fact that the defense system in it is incomplete, which allows protease enzymes to cause fatal damage to this instar. Likewise, the effectiveness increased with the increase in the concentration of protease due to the increase in its role in the effect and the increase in the percentage of death, as the insect's immune system can defend the body only at weak concentrations, while at high concentrations the immune system loses its efficiency (37).

The weak effect of the protease enzyme on the fourth and seventh instars when treated in contact is due to the fact that the building of chitin in these two instar reaches its maximum degree, especially in the larvae of the last instar and at the pre-pupae stage and the white pupae stage, and once again the formation of chitin doubles before the emergence of adults by 4-6 days (6).

Confirmed Ramarao *et al.*, (34) in his study that the larvae of Lepidoptera showed sensitivity to toxic proteins and accelerated death rates in the larvae of the great wax moth in the seventh larval instar by direct injection.

Harrison and Bonning (19) and Haq *et al.*, (18) show that proteases are reasonable candidates for use as insecticidal agents. Proteolytic enzymatic activity can target and destroy essential proteins and tissues to death. Proteases that have not evolved to play Dura-as-toxin can have an insecticidal effect when present at the wrong time, wrong place, and/or the wrong amount within the insect.Protease sources are diverse (viruses, bacteria, fungi, plants, and insects) and have toxicity towards insects. The sites of toxic activity of proteases range from the gut Median of the insect to the body cavity hemocoel to the epidermis.

Evidence for the effectiveness of the protease enzyme in resisting insect pests has been the use of many growth regulators such as hexaflumuron, hydrobin and chloroforazone, which depend in their mechanism on digestive enzymes such as protease, amylase and lipase (25).

Likewise, many fungi that are used in the biological control of insect pests of Lepidoptera depend in their mechanism on chemical penetration with digestive enzymes, especially proteases, as is the case in the fungus *Metarhizium anisopliae* (6; 16).

4. conclusions

The results showed that all treatments used in contact (direct spraying) except for distilled water achieved a complete mortality rate of 100% for the first larval instar, while in the fourth and seventh larval instars, the alcoholic extract of soft tissues maintained a high mortality rate that reached (100% and 70.00%, Compared to the rest of the treatments, where it was significantly superior to it, except for its effect on the seventh instar, which had non-significant differences with the pesticide, The efficiency of the aqueous extract of shells and protease decreased on the fourth and seventh larval instars compared to their effect on the first larval

instar. The current results for the used concentrations showed a high efficiency of 100% for all the concentrations used on the first larval instar, then the effect of these concentrations decreased on the fourth and seventh instars, with reference to an increase in the effect with an increase in the concentration in these two instars.

5. References

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