Evaluation of the efficacy of extracts of two types of Mollusca to control the eggs of the Greater wax moth Galleria mellonella (L.) (lepidoptera: pyralidae)

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Abstract
The extracts of two types of molluscs were used, one of which was prepared in the laboratory represented by 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}, and conducted GC-MS analyzes of soft tissues and shells, and the analyzes of oxides and minerals by X-ray fluorescence technology of the shell extract. The other one is a commercial preparation which is a protease enzyme of *Achatina fulica* "White Jade" 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 egg stages of the greater wax moth *Galleria mellonella* (L.) and to compare the results, a new insecticide was used to control Lepidoptera insects known as Belt$_{480SC}$ (Flubendiamide) at concentrations (0.1, 0.2, 0.3 and 0.4) ml.L^{-1}. The soft-tissue alcoholic extract and aqueous extract of shells and their mixture were significantly superior in killing the eggs of the greater wax moth by (100%) at the highest concentration of 40 mg.ml^{-1} for the two extracts and at the concentrations of 30 and 40 mg.ml^{-1} for the mixture, compared to a lower mortality rate of which it reached (86.66%) for the protease at a concentration of 10 mg.ml^{-1} and (90.66)% for Belt$_{480SC}$ at a concentration of 0.4 ml.L^{-1}, and the relationship was positive between the concentrations used for the treatments and the mortality rates of eggs.
Keywords: Galleria mellonella, Protease, Mollusca, Egg, Flubendiamide.

Introduction

The greater waxworm, *G. mellonella* (L.) belonging to the family Pyralidae, is a severe pest with a global reach and is considered one of the permanent and important economic pests that attack wax combs and pollen inside honey bee hives and in storage. The risk of infection with this pest increases at the end of the honey season and tire storage. beekeepers all over the world suffer significant economic losses as a result of the wax moth attacking stored wax frames and weakened bee colonies, so this pest has received more attention as a model organism for toxicological and biological control research (21; 25; 8).
After mating, the female begins laying eggs in batches of 50-150 eggs, luring them together in order to stick firmly and firmly to the surfaces they are placed on (23,15) indicated that the number of eggs per female ranges from 175-355 eggs, dimensions ranging from 0.44-0.47 mm in length and 0.29-0.39 mm in width (22; 25; 17; 35; 32; 18)
In this study, Belt$_{480sc}$ was used, which is one of the modern insecticides belonging to the new chemical group Benzene Dicarboxamide or Phthalic Acid Diamide, and the chemical group is characterized by the strength and speed of effectiveness against a wide range of Lepidoptera pests. economically important, including resistant strains, contains 480 g.l^{-1} of the active substance flubendiamide, and its composition is in the form of a water-suspension concentrate or white crystalline powder, works by contact and ingestion, with high permeability (penetration), with a lipophilic nature, Broad spectrum against all stages of Lepidoptera larvae (15).
Also, extracts of two species of nymphs were used: *P. euphraticus*, which belongs to the Bivalvia class, and *A. fulica*, which belongs to the Gastropoda class. The benefit of these invertebrates comes from the presence of a group of active components in the form of carbohydrates, proteins, minerals, lipids, 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 (2, 6, 14). From here, the study included the use of extracts of two types of molluscs (oysters and snails) in the control of eggs 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.

Materials and Methods

Preparation of extracts

Alcoholic extract of the soft tissues of oyster

The outer shell was opened, the soft part was

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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 (6), 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, 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 (27; 9).

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 (7; 26).

The aqueous extract was prepared according to the method of (4, 16) 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 (27; 9).

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.

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

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.

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.

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) $mg.ml^{-1}$.

Effect of different treatments on eggs of the greater wax worm.

To get eggs not more than 24 hours old, prepare 3 cages with dimensions of 30 x 30 x 30 cm. The base is made of wood and the front is made of muslin cloth, with a sliding zipper in the shape of an inverted U (to control opening and closing) and the rest of the fronts are made of wire mesh, then placed Small pieces (approximately 1.5 - 2 cm) of dark wax sterilized by cooling previously were placed in petridishes, then these dishes were placed inside the cages, after which four females and four males, aged no more than 24 hours, were transferred to each cage from the laboratory colony, and placed on the part The top of the cage (on the wire clamp) is a piece of cotton saturated with a 10% sugar solution to feed the adults, taking into account its

replacement every two days. The cages were placed in sterile cupboards and at laboratory temperature with complete darkness. Wax pieces were examined daily under a microscope for the purpose of collecting newly laid eggs sandwiched between the wax membranes and less than 24 hours old.

After collecting the eggs, three sterile petridishes with a diameter of 9 cm and a height of 1 cm were prepared, representing three replicates for each treatment, and 100 eggs were placed inside them for each replica, which were counted under a microscope, then the eggs were sprayed with 3 ml of each of the pesticide concentrations and extracts, separately, using a hand sprayer with a capacity of 100 ml, from a height of 15 cm, vertically. While control treated eggs were sprayed with distilled water only, all replicates were placed in dark, sterile tanks, except for the protease treatment, it was carried out in the incubator at 25 ± 2 °C and 12 light hours.. After the sixth day of laying the treated eggs, the daily examination begins and until the completion of the hatching process within a period of 10 days, then the mortality rate is calculated.

Statistical design and analysis

Statistical Analysis System -SAS (31) 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).

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.

Table ((1): The effect of the interaction of concentrations of pesticide Belt$_{480SC}$, alcoholic extract of soft tissue , aqueous extracts of shells and their mixture of oyster *P. euphraticus* and protease enzyme of snail

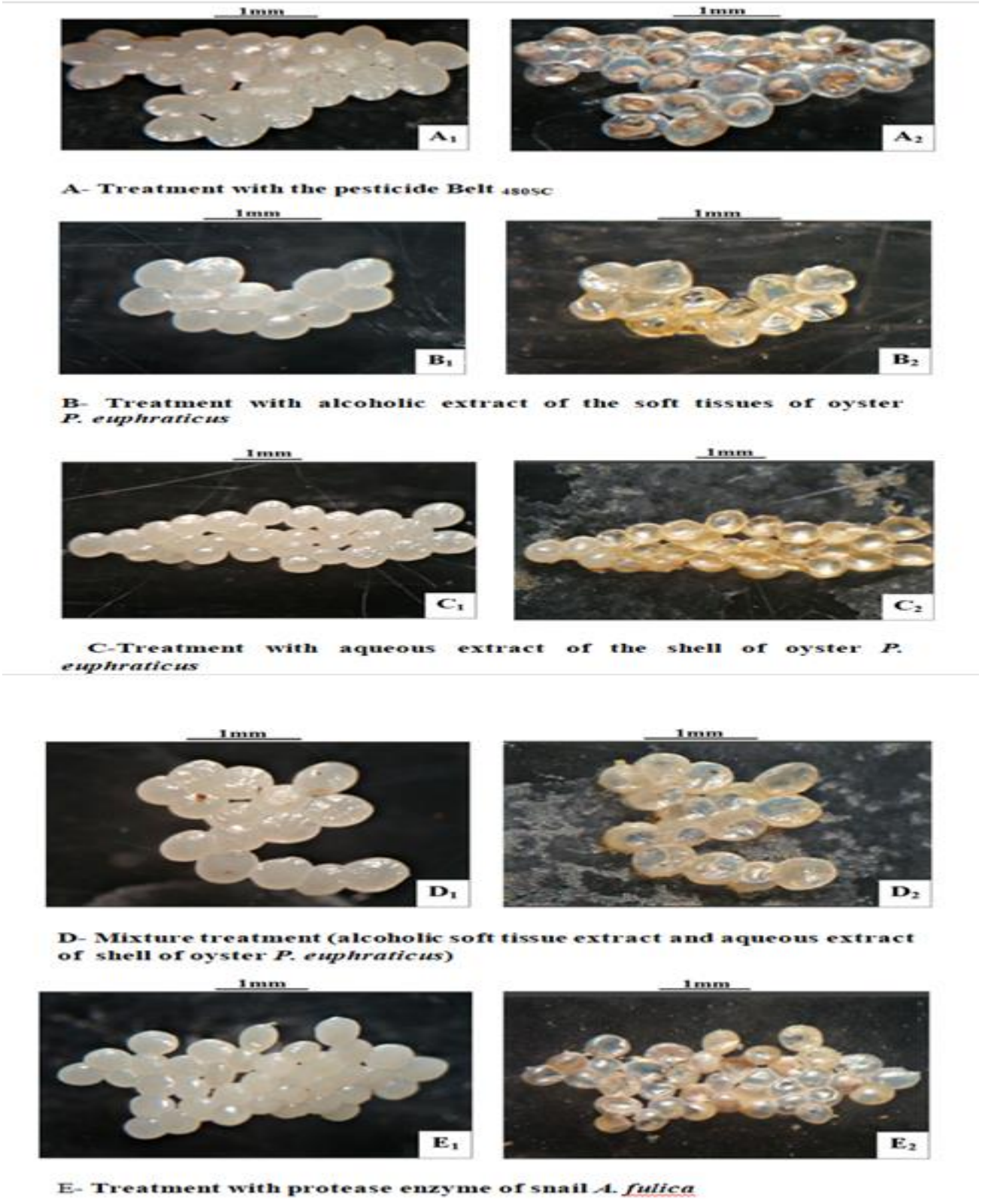
Concentrat-ions	Percentages of egg deaths					Concentr-ation rate
	pesticideBelt 480SC	alcoholic extractof soft tissue	Aqueous extract of shells	mixture	protease enzyme	
Control	0.00	0.00	0.00	0.00	0.00	0.00
I	77.00	77.33	73.33	90.66	67.33	77.13
II	79.33	85.33	81.00	97.66	74.66	83.60
III	82.66	98.66	93.00	100	79.33	90.73
IV	90.66	100	100	100	86.66	95.47
Treatments rate	65.93	72.27	69.47	77.67	61.60	---
LSD 0.05	7.94 * Treatm =		7.94 * Concen =		12.87 = * Overlap	

The results of Table (1) showed the effect of different treatments on the rate of egg mortality of the greater wax moth, as the treatment of mixing (the mixture) between the alcoholic extract of soft tissues and the aqueous extract of oyster shells excelled in increasing the efficiency of killing the eggs of the greater wax moth at the age of 48 hours, compared to the effect of each extract separately. As the death rate in each of them reached its peak at the highest concentration, which was all superior to the two treatments of the pesticide Belt $_{480SC}$ and the protease enzyme. The statistical analysis confirmed the significant differences between the treatments, and a direct relationship was found between the concentrations of the used treatments and the death rates, as the highest percentage of death was recorded as 100% in the mixing treatments and extracts at the two concentrations of 30 mg.ml^{-1} and 40 mg.ml^{-1}, respectively, while the lowest rates were recorded mortality was 90.66% and 86.66% in the two treatments of Belt $_{480SC}$ and protease enzyme, respectively, at their highest concentration, compared to the treatment at zero concentration (distilled water), as the mortality rate reached zero%.

A. fulica on the death rates of 48-hour-old eggs of the greater wax moth G. mellonella.

* 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}).



Picture(1): Pictures of the eggs of the greater wax moth (Galleria mellonella)(L). with different treatments.1 Before the treatment, 2- After the treatment.

The superiority of the treatments of the alcoholic extract of the soft tissues of oyster and the aqueous extract of their shells and the mixture between them is attributed to the toxic effect of the active substances of the extracts, which are alkaloids, glucosides, saponins and terpenes based on the results of GC-MS analysis, in addition to many different metals and their oxides for the extract of shells based on X-ray fluorescence (XRF) technology.

The results and photo (1-B2 and 1-D2) show the ability of each of the alcoholic extract of soft tissues and the mixture to penetrate the egg shell and prevent the formation of embryos in treated eggs at the age of 48 hours, and this is due to the fact that the active substances in both treatments above contributed effectively to increasing the rate of inhibition of eggs the result is either an increase in enveloping the egg shell and preventing it from

breathing completely, or an increase in the entry of toxic compounds into the eggs and preventing the formation of embryos (10).

While the photo (1- C2) shows the ability of the aqueous extract of shells to dry eggs and thus prevent the formation of embryos, which explains that in addition to the extract containing effective compounds, it also contains silicon dioxide (silica) SiO2, which works by adsorption / absorption of fats By direct contact, when the waterproof film is lost, the eggs dry up (28 , 34). Thus, these extracts were superior to the chemical pesticide and protease enzyme treatments.

In general, the high effects of alcoholic extracts of the soft tissues of oysters and aqueous extracts of shellfish can be explained because they contain three main groups depending on their chemical composition, which are alkaloid compounds, phenolic compounds, and terpenoids compounds.

Also, the effect on eggs is due to the chemical composition of the tissues and shells of oyster, as there are many chemical groups that have an effect that prevents feeding or laying eggs for many insects, such as mineral salts (inorganic) such as sodium, calcium, potassium, and copper if they are found in high concentrations that the insect does not accept, and also Compounds containing sulfur and some acids and bases, as well as aromatic hydrocarbons, alcohols, aldehydes, ketones, amino acids, cormarines, alkaloids, sterols, terpenes, and the effect depends on the concentration of the substance. Sometimes the low concentration of the same substance is activating, while the high concentration is inhibiting, even at a high concentration, the inhibitory effect of the substance is rarely general for all insects, and so far it is difficult to attribute these effects to one chemical substance, or for the same substance to have a general effect on all insects (13).

It is possible to compare the results of this study with previous studies in terms of effect only and with the difference in the nature of the extract, the current results are consistent with many studies that found the role of the extracted materials in reducing the number of eggs, the destruction of eggs, and the destruction of different life stages of the greater wax moth insect, Abdul- Jabbar (1) found a decrease in the hatchability of eggs when treated with eucalyptus extract, which contains a high percentage of glycosides and mineral elements, to reach 1.33 at a concentration of 10%.

Likewise, Al-Amiri (5) found that the volatile oils extracted from caraway, cumin and thyme seeds, which contained glycosidic substances and terpenes similar to Those found in tissue and shell extracts of oyster, had an anti-egg-laying effect, prevented the eggs from hatching, and prolonged the incubation period of the eggs.

As for Hadi (20), she confirmed that plant extracts and regular and nano oils (for cinnamon, turmeric and cardamom plants) led to an increase in egg mortality rates, as nano oils outperformed regular oils and boiled water extract, reaching 76.60% compared to 32.66 and 25. 84% respectively. As for the type of plant, the cinnamon plant outperformed the turmeric and cardamom plants, reaching 52.09% compared to 46.76 and 47.59% for each of them, respectively, and attributed the reason to the fact that the cinnamon plant contains phenolic, aldehyde and ester compounds, while the turmeric contains flavonoids and resins, while the cardamom contains glycosides Alkaloids, saponins, tannins.

The current results showed that the higher the concentration of the extracts used, the greater its efficiency in killing the eggs of the greater wax moth, and the highest concentration of 40 $mg.ml^{-1}$ of these animal extracts achieved a complete death rate of 100%. It should be noted that the differences with the third concentration of 30 $mg.ml^{-1}$ were non-significant for the three extracts. Likewise, the effect of all used concentrations of the mixture of alcoholic soft tissue extracts and aqueous shells had similar effectiveness, as there were no significant differences between them and ranged between 90.67-100.00%, and therefore it is recommended Using this mixture for its high efficiency in killing eggs.

This opens the way for the replacement of chemical pesticides with compounds that are safer for the environment, as the use of pesticides in recent years in pest control programs has led to an imbalance in the environment, an outburst of the pest and an increase in the emergence of resistant strains, in addition to the harmful effect on non-target organisms (vital enemies). In addition to the direct toxic effects on users of these pesticides. Therefore, it was necessary to search for safe alternatives to these pesticides, the most important of which are extracts as a primary and basic means to protect production and the environment from pesticide contamination (3; 29).

As for the eggs treated with the $Belt_{480SC}$ pesticide (photo 1-A2), it shows the failure of the effective substance of the pesticide to penetrate the egg shell and the embryos continued to grow and became small, complete larvae curled around themselves, but they faced great difficulty in breaking and tearing the egg shell and exiting it naturally, and thus the embryos became difficult and died inside the egg and the increase The effect on the egg shell by increasing the concentration of the active substance of the pesticide. The reason is attributed, according to the explanation of Batish *et al.* (11), to the entry of the active substance (of the pesticide) from the hilum opening and causing a biological disturbance inside the eggs, which leads to the inability of the larvae to tear the membrane of the eggs and exit normally. It is possible to compare the results of this study with previous studies in terms of effect only and with the difference in the nature of the extract.

This is consistent with the findings of Taqi (33) when treating the eggs of the greater wax moth with Avaunt150SC and the inhibitor of chitin formation Match050EC, as the hatching rate reached 0%, while

Karim (24) used the Karate pesticide on the eggs of the pest, and the hatching rate reached 0% as well. While eggs treated with protease enzyme (PH 6.1) (photo 1-E2) show medium permeability of the enzyme through the egg shell without affecting the shell itself, which allowed some of them to complete embryonic formation and hatch naturally, while the other part became difficult in the middle stages of formation, the effect increases with increasing concentration. The protease enzyme belongs to an important class of enzymes known as hydrolytic enzymes that catalyze the hydration of proteins (23). The reason for the effect may be due to the principle of active toxicity present in the extracts (such as protease enzyme) that can block the micropyle region in the placenta, leading to the death of the fetus due to depletion of oxygen for the purpose of respiration, or the interference of the extract with normal embryonic development by preventing hormonal and chemical processes fetal vitality (30). Al-Moussawi (8) mentioned that the enzyme caused death rates ranging between 31.78 and 62.88% for the eggs of the great wax moth, the protease enzyme showed specialization towards many reaction materials, which indicates the possibility of using it in biological control of many insect species during the autumn and spring seasons, and it cannot be used in summer due to its lack of stability at high temperatures.

Conclusions

The treatments of the alcoholic extract of soft tissues and the aqueous extract of oyster shells and the mixture between them achieved the highest rates of egg destruction reaching (100%), outperforming with significant differences over the two treatments of the standard pesticide Flubendiamide and the protease enzyme. The effect of these extracts increased with increasing concentration, and the highest concentration of the used treatments achieved the best efficacy against eggs.

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