

Utilizing *Rosmarinus officinalis* Nanoparticles for Eco-friendly Control against Two Cotton Bollworms: Investigating Biological, Biochemical, and Histological Effects

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ABSTRACT



A growing concern has emerged regarding the extensive use of pesticides. This study aimed to explore an eco-friendly alternative by utilizing silver nanoparticles (AgNPs) derived from *Rosmarinus officinalis* L. leaves, which were evaluated against *Earias insulana* and *Pectinophora gossypiella* UV-VIS spectroscopy analysis of the Rosemary-AgNPs exhibited an absorption at wavelength around 403 nm, characteristic of silver, confirming successful nanoparticle synthesis. HPLC analysis identified twenty-six compounds from *R. officinalis*, contributing to its chemical composition. Additionally, TEM imaging revealed the spherical morphology of the AgNPs with an average diameter of 20-22 nm. The LC₅₀ values for *P. gossypiella* and *E. insulana* were determined to be 18.655% and 16.75%, respectively, indicating the potency of the AgNPs against these pests. Furthermore, the significant prolongation in the developmental stages of both insects, with total immature stages taking 33.83 and 34.9 days for *P. gossypiella* and *E. insulana*, respectively compared to the untreated group 21.9 and 24.3 days. Biochemical analysis demonstrated a marked decrease in total protein and carbohydrate levels, reducing to 48.5 and 11.5 mg glucose/g, opposed to 114.0 and 29.5 in the untreated, respectively. Conversely, a notable increase in total lipid content, reaching 12.0 mg/g in the *P. gossypiella* strain, compared to 7.5 in the untreated group. However, in *E. insulana*, there was a substantial reduction to 2.3 mg/g, down from 4.1 mg/g in the untreated group. Additionally, the cuticle and midgut levels, the treatments led to histological irregularities in both tested insects when compared to the untreated controls. The findings emphasize the viability of eco-friendly alternatives, promoting further exploration and utilization of nature-derived solutions in pest control over conventional chemical insecticides.

Keywords: *Earias insulana*; Nano particles; *Pectinophora gossypiella*; Plant extract; *Rosmarinus officinalis*.

INTRODUCTION

Earias insulana (Boised) and *Pectinophora gossypiella* (Saunders) and are widely recognized Lepidopterans as prevalent insects, known for their destructive impact on various crops. These two species, belonging to the Nolidae and Gelechiidae families, respectively, exhibit a remarkable adaptability to diverse environments worldwide. Since as early as 1916, their presence and damaging effects on crops, particularly cotton, have been well-documented. In Egypt and various regions, the larvae of these pests continue to pose significant threats to a wide range of agricultural products (Hariprasad, 1999; Kandil, 2001; AbdAllah *et al.*, 2023; Abd- ElAzeem *et al.*, 2023).

Cotton bollworms are significant agricultural pests that inflict substantial damage to cotton crops on a global scale. Conventional pest management strategies, predominantly reliant on chemical insecticides, have elicited concerns due to their detrimental impacts on the environment, human health, and non-target organisms. Consequently, there exists a pressing demand for environmentally friendly and sustainable alternatives for effective pest control. In response to this need, researchers have investigated diverse eco-friendly methodologies, including the utilization of natural plant extracts. Green insecticides that are biologically derived and produced from plant extracts offer a viable and long-term approach to controlling

pests like *Pectinophora gossypiella* and *Earias insulana* (Li *et al.*, 2021). One practical method in pest management that has arisen is the application of plant extracts as green chemistry-based alternatives (Rónavári *et al.*, 2021).

Rosemary, *Rosmarinus officinalis* L., extracts have gained significant attention for their potential in insect control due to their natural insecticidal properties. These extracts are derived from the plant leaves that contain a variety of bioactive compounds, including essential oils, phenolic acids, and flavonoids, which contribute to their insect-repelling and insecticidal effects (Micić *et al.*, 2021).

Nano-formulation especially silver nanoparticles enhances efficacy of plant extracts, allowing for lower application rates, thereby reducing the overall environmental impact (Mena *et al.*, 2019). Furthermore, counterparts align with the principles of integrated pest management (IPM), promoting a holistic approach to pest control. This method not only targets the pests directly but also considers the broader ecosystem, preserving natural predators and minimizing disruptions to the ecological balance, this approach marks a crucial step towards a more resilient and ecologically balanced agricultural system (Kandil and El-Shennawy, 2018; Pathipati and Kanuparthi, 2021). Therefore, the aim of our study was to elucidate the effect of using *Rosmarinus officinalis* extract-Nanoparticles as eco-friendly control on two cotton

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bollworms, with respect to the worms' biology, biochemistry, and potential for histological changes.

Materials and Methods

Used Plant

Rosmarinus officinalis L., commonly known as rosemary, is taxonomically classified within the genus *Rosmarinus*, belonging to the family Lamiaceae. The genus *Rosmarinus* includes a small group of evergreen shrubs that are widely recognized for their aromatic leaves and culinary use (Andrade *et al.*, 2018; de Macedo *et al.*, 2020).

Preparation of peels powders

Fresh leaves of Rosemary (*Rosmarinus officinalis*) were collected in August 2021 from a natural field in Zagazig - Sharkia, Egypt and by a taxonomist in the department of Botany, Faculty of Science, Zagazig University, Egypt. Then washed with distilled water and dried yield (250 gm.). The powder was stored at 4°C for further analysis.

Extraction of rosemary leaves

Fresh dried leaves of Rosemary (250 g) in 500 mL distilled water and putted in water bath at 60 °C for 1 hr. The solution left to cool, and then filtered with Whatman filter paper to remove plant debris and obtain clear green extract, a rotary evaporator apparatus was used to remove the solvent, yield extract was 3.1 g. The dried plant crude extracts kept in refrigerator for further analysis (Abaza *et al.*, 2015).

Chemicals Reagent

All requirements solvents were purchased from Merck (Germany) and standards of Phenolic compounds were obtained from Sigma-Aldrich for identification and quantification.

Chemical constituent of rosemary leaves extract

High Performance Liquid Chromatographic (HPLC)

HPLC analysis was carried out in National Research Center, (NRC), Giza, Egypt, using a Shimadzu Class-VP HPLC system with a computer-controlled system containing upgraded Class-VP 5.03 software. Separation was carried out on a reversed phase column LiChro-CART RP-18 (Merck, Darmstadt, Germany, 12.5 cm x 0.4 cm, particle size =5 µm), using mobile phase of water/formic acid (19:1v/v) (solvent A) and methanol (solvent B) at a constant solvent flow rate of 1ml/min. The following gradient was used, according to the method of (Yao *et al.*, 2003). To support the identification of the differential kind of phenolic flavonoid and saponin compounds (Zhang and Ye, 2009). The injection volume was 20 µL and peaks were monitored at λ 280 nm peaks were identified by comparison of their relative retention times with those of authentic standards analyzed in the same conditions.

Biosynthesis of silver nanoparticles of Rosemary extract (R- AgNPs)

Silver nanoparticles were created by combining silver nitrate (AgNO₃), distilled water, and aqueous extract of Rosemary leaves, in co-operation with the Faculty of Science, Zagazig University, Egypt. Silver nanoparticles were prepared from aqueous silver nitrate

using a simple green route and rosemary leaves extract as a reducing and capping agent (Al-Khafaji *et al.*, 2023). The AgNPs were prepared from Rosemary leaves extract using the previously mentioned procedure with slight adjustments (Labeeb *et al.* 2023). The silver nitrate (0.034g) was added to 200 mL of double distilled water to make a 1 mM silver nitrate solution. 200ml of 1 mM AgNO₃ solution was put in 200 mL beaker, then, 100 mL of rosemary extract solution was dropped from burette and heated at room temperature for 24 hrs using a hot plate with a magnetic stirrer (1000 rpm). As soon as, the extract solution was added, precipitate formed in the solution as dark brown color. Enough precipitate was found after the addition of 100 mL of solution. The solution rested until reaching room temperature. It was separated using high-speed centrifugation at about 8000 rpm. Finally, the firm mass was dried in an oven set to 700 °C. The full drying of this solid mass produced a black-colored material (30 mg), which was powdered in mortar and sampled for characterization.

Characterization of silver nanoparticles

UV-Visible spectra spectrophotometer

Periodic scans of optical absorbance between 300 and 700 nm were conducted using a double-beam UV-visible spectrophotometer (Carry 100 with tungsten halogen light sources) to investigate the reduction of silver ions by a methanolic extract. The biosynthesized AgNPs solution was collected at room temperature after various time intervals (15, 30, 45, 60, and 90 min). UV-Visible spectroscopy was performed at the Faculty of Science, Zagazig University, Egypt.

FTIR spectroscopic analysis

FTIR measurement was carried out to identify the functional groups responsible for capping and reducing agent for the silver nanoparticles. AgNPs synthesized using aqueous extract of rosemary leaves were confirmed by ATR FTIR using Bruker Vertex 80 (Germany) combined platinum diamond ATR, comprises a diamond disk as that of an internal reflector in the range 4000-400cm⁻¹ with resolution 4 cm⁻¹, refractive index 2.4.

Transmission Electron Microscope (TEM)

TEM analysis was performed in National Research Center, (NRC), Giza, Egypt. The analysis was done using Philips (technai 10). Thin films of sample were made on a carbon coated copper grid by simply dropping a very small amount of sample on the grid, excess solution was removed through blotting paper, and the film on the TEM grid allowed drying in an incubator. In this technique, an electric beam is passed through an ultra-thin specimen, communicating with the specimen as it passes through. The presence of electrons passing through the specimen results in the formation of an image. An imaging system is used to magnify and focus the image.

Insect pests

Rearing technique

Larvae of both susceptible strains spiny boll worm (SBW) and pink bollworm (PBW) (0 to 6h old) were used in the experiment. The two strains were obtained

from a laboratory culture that had been raised for almost 20 generations on a control diet with no use of pesticides, as previously explained by (Rashad and Ammar, 1985; Amer, 2015). All culture Bollworms Research, Zagazig branch, Department, Plant Protection Research Institute.

Tested concentration of R-AgNPs

Serial aqueous dilutions from *R. officinalis* extract nanoparticles (R-AgNPs) were freshly prepared to run the mortality test. The tested concentrations were 40, 20, 10 and 5 %.

Toxicity assessment

An *in vitro* assay was applied to determine the potential toxicity (LC₂₅, LC₅₀, and LC₉₀ % values) of; naturally synthesized R-AgNPs against 1st larval instar of *E. insulana* and *P. gossypiella*. The prepared concentrates were sprayed on the surface of an artificial diet (3g) that was placed into Petri dishes. Three replicates were used for each concentration; each consisting of 30 newly hatched larvae of each tested insect. Both, *E. insulana* as well as *P. gossypiella*, tested larvae were allowed to feed on the treated diet for each concentration. The same numbers of larvae were left without treatment as untreated. Both treated and untreated larvae were kept under constant conditions of 26±1°C and 65±5 % RH. Both treated and untreated larvae were inspected after 24 hrs to 7 days and the alive and dead numbers of larvae were recorded. All prepared concentrations were used to estimate LC₂₅, LC₅₀ and LC₉₀ values for R-AgNPs against newly larvae of *P. gossypiella* and *E. insulana*.

Statistical Analysis

For all inspection days, larval mortality percent at all concentrations was recorded and corrected according to Abbott (1925). The mortality percentages for different concentrations of the tested compound were conducted to estimate LC₅₀ and LC₉₀ values with their fiducially limits by Probit (proban) analysis software according to Finney (1971). Differences in the biological aspect and biochemical parameters were analyzed using One-way analysis of variance (ANOVA), Duncan's Multiple range test was used to detect the significant difference (LSD) was used as a post-hoc test.

Bioassay

Effects of R-AgNPs on Larvae Development

The LC₅₀ value of *R. officinalis* extract nanoparticles was sprayed on the surface of an artificial diet (3 g/Petri dish). The larvae of both *E. insulana* and or *P. gossypiella* were allowed to contact and feed on the sprayed diet and then kept under a constant condition of 26±1°C and 75±5 % RH for 3 days). The survivors' larvae from *P. gossypiella* and/or *E. insulana* were transferred individually (by a hairbrush) to the diet tubes (2x7.5 cm) each containing about 2 g of artificial diet. A similar group of untreated larvae was used as a control check. All tubes were labeled, capped with cotton, and kept in an incubator under the previous constant condition for daily inspection until pupation.

Estimation of some biological measurements

Newly hatched larvae of *E. insulana* and *P. gossypiella* were treated with the respective LC₅₀ con-

centrations of the tested compound, while an untreated control group was treated with water only. Several biological para-meters were observed and recorded on a daily basis, including the following:

For the larval stage

Number of dead larvae, percentage of malformed larvae, and duration of the larval stage (in days) were recorded.

For the pupal stage

Duration of the pupal stage (in days), percentage of pupal mortality and percentage of pupal malformation were documented. Additionally, the total duration of the immature stages (combined larval and pupal stages) was recorded.

Biochemical and histological studies

Sample reared and collected for biochemical and histological measurements

For biochemical or histological assay, the LC₅₀ value of extract rosemary plant tested compound were sprayed on the surface of an artificial diet (3 g) that was placed into Petri dishes. Three replicates were used for each concentration, each of 30 newly hatched larvae of each tested insect were allowed to contact and feed on the treated diet for three days. A similar group of untreated larvae was used as a control check. After 7 days of treatment, alive larvae of all tested insects were collected biochemical and/or histological examinations.

Biochemical measurements

Sample preparation

The current experiment was created to investigate the effects of treatment on total soluble amounts of each protein, lipid, and carbohydrate as well as the activity of enzymes that hydrolyze carbohydrates in total homogenates from both insects. The treated and untreated *P. gossypiella* and/or *E. insulana* larvae (6-7 larvae/control to 32 larvae/treatment) after 7 days of treatment were used in the sample preparation process.

Samples preservation:

Samples were homogenized in distilled water. Centrifuging the homogenates was done at 5000 rpm and 5 °C. When used for biochemical tests, the supernatants were maintained in a deep freezer at a temperature of 20 °C. All processes were completed at the Physiological Department of the Plant Protection Research Institute. The colorimetric analysis of total soluble protein, total lipids, and total carbohydrate was estimated for *P. gossypiella* or *E. insulana* larvae in the total homogenate according to the methods of Brad-ford, 1976; Knight, 1972; Cromppton and Birt, 1967. According to (Ishaaya, 1971) the digestion of amylase, trehalase, and invertase enzymes, was determined.

Histological measurements:

The histological studies were conducted on the treated larvae (the newly hatched larvae that subjected to RNP for 10 day) of *P. gossypiella* and *E. insulana* that contacted and fed on the LC₅₀ of the RNP, compared to the untreated larvae after 10 days from treatments, samples were fixed in 10% formalin followed by the rest of the histological stages of

making sectors. The obtained tissue sections were collected on glass slides and then deparaffinized, stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft *et al.* 1996).

RESULTS

Phenolic and flavonoid compounds of rosemary leaves extract using HPLC

The investigation of phenolic acids and flavonoids compounds of rosemary leaves extract data was recorded at (Fig. 1 and Table 1), showed presence of 26 phenolic compounds compared to standard samples where, the highest concentrations were, methyl eugenol (15.15%), carnosic (13.81%), gallic acid (10.04%), rosmarinic (9.42%), rosmanol (8.16%) and hispidulin 7-glucoside (5.64%).

Biosynthesis and mechanism of AgNPs formation

The biosynthesis of nanoparticles was demonstrated by the color shift that was seen from pale yellow to brown when the extract was introduced to the silver nitrate solution. The synthesis of silver nanoparticles (AgNPs) involves a complex mechanism that is influenced by the presence of bioactive compounds such as phenolic acids and flavonoids. These compounds act as capping and reducing agents during the synthesis process and have the ability to donate electrons, which is crucial for the reduction of silver ions (Ag^+) to silver nanoparticles (AgNPs). In addition, during the synthesis process, the carboxyl and hydroxyl groups present in the bioactive compounds play a role in forming a protective coating on the surface of the silver nanoparticles

Characterization of the rosemary silver Nanoparticles (RNPs)

UV-Visible Spectrophotometer Analysis

The UV-Vis spectroscopy of the synthesized nanoparticles revealed an absorption peak at 403 nm. The extract from rosemary leaves facilitated the synthesis of silver nanoparticles by providing suitable surface plasmon resonance (SPR) conditions. The presence of peaks near the visible spectrum at 403 nm indicates the formation of high-quality AgNPs (Fig. 2).

FTIR Spectroscopic analysis

FTIR measurement was carried out to identify the functional groups responsible for capping and reducing agent for the silver nanoparticles. FTIR spectrum of RNPs (Fig. 3). IR (Vmax , cm^{-1}): 3293.74 (-OH), 1557.05 (C=O amide), 1406.77 (C-C aromatic), 1393.88 (NO_3) and 1257.20 (C-O ether), the peak at 410.42 cm^{-1} can be attributed to the presence of more important quantity of AgNPs.

Biological measured parameters

Time observation and mortality of 1st instar larvae

The data recorded in Table (2) showed that on the first day, the RNPs treatments did not have a significant effect on the mortality of larvae from both strains across all concentrations tested. Since a mortality of less than 50% is generally considered ineffective. As the time proceed, the results indicated

that the extract's efficacy caused a progressive daily increase in the mortality rates. Furthermore, the RNPs treatment at the 40% concentration was able to achieve 100% mortality of the insects within 8-14 days after the initial treatment application.

The data recorded in Table (2) showed that on the first day, RNPs was not at all proper effect on both strain larvae with all concentrations. Since a mortality of less than 50% is ineffective, it was shown that extract efficiency caused a progressive daily increase in mortality. Also, RNPs at 40% could kill all insects after 8-14 days from treatment.

The potency of (RNPs) tested against the two pests (*E. insulana* and *P. gossypiella*) represented as mortality number at different concentrations (40, 20, 10 and 5%). It increased as the concentrations increased. According to the current study, when the larvae were contact and feed with the RNPs, the numbers mortality decreased gradually 58, 40, 28 and 20 larvae, respectively, compared to 4.0 in untreated *P. gossypiella* and 60, 45, 33 and 10, respectively, compared to 6.0 in untreated *E. insulana* larvae. In addition to the toxic effect and the repellent effect of the tested compound leads to a slow or even prevent feeding process which subsequently has a high adverse effect required supplementary necessary for energy production and survival.

Toxicological evaluation of RNPs

According to data in Table (3) *P. gossypiella* was less susceptible to (RNPs) LC_{50} than *E. insulana* which recorded 18.65 and 16.757%, respectively.

Time development of larval and pupal stages

As noticed in Table (4), it is obvious that increase in the time required for developmental larval and pupal stages, as it recorded by 21.53 days/larvae of *P. gossypiella*, while it increased to 23.5 days when *E. insulana*, resulted from neonate larvae treated with LC_{50} of RNPs, compared with 14.6 and 16.3 days/untreated larvae *P. gossypiella* and *E. insulana*, respectively, In addition, the average of pupal duration increased to 12.3 and 11.6 days for the previous compound, compared to 7.8 and 8.3 days/ pupal stages in *P. gossypiella* and *E. insulana*, respectively, in untreated. The results acquired and shown in Table (4) revealed a considerable increase in the amount of time needed to complete the total immature stages in both insects *P. gossypiella* and *E. insulana*, it reached to 33.83 and 34.9 days respectively, resulted from treated newly hatched larvae with LC_{50} of RNPs, compared with 21.9 and 24.3 days in the untreated check.

Transmission Electron Microscope (TEM):

TEM is used to assess the morphology, shape, and size of nanoparticles. TEM images of synthesized AgNPs at various magnifications are shown (Fig.4 and 5). The size of the synthesized AgNPs in the average diameter is ranged from 20-22 nm. The AgNPs were spherical shaped.

Effect of RNPs (LC_{50}) on body weight and malformation

The data presented in Table (5) clearly showed that the body weights of the larvae and full-grown stages were significantly ($p \leq 0.001$) reduced. Larvae

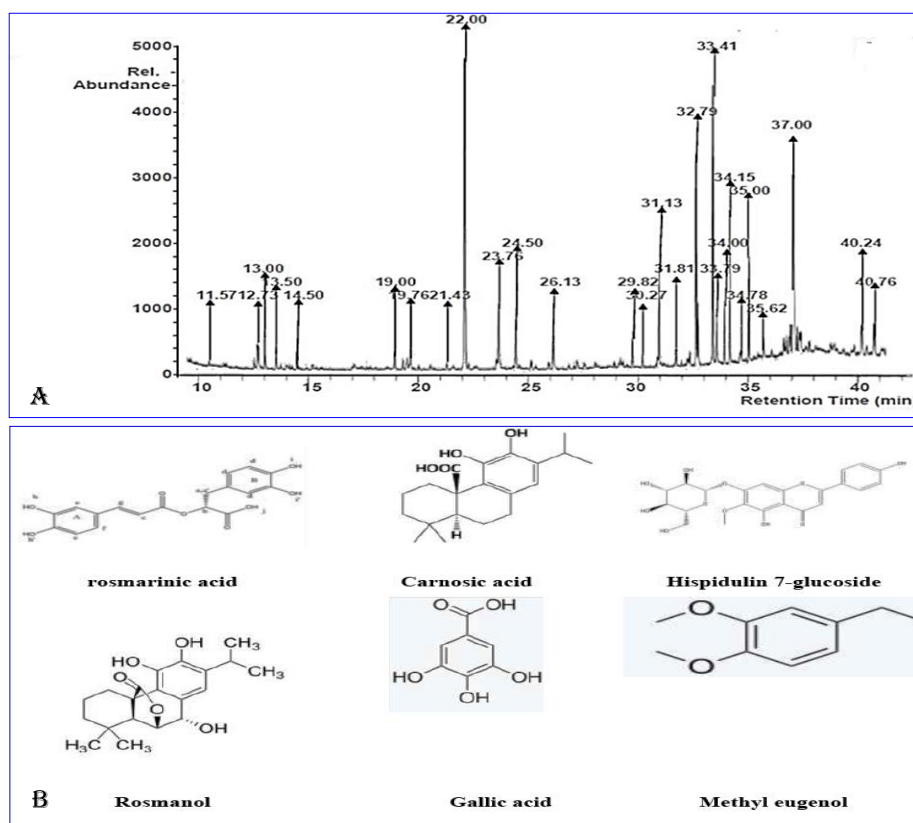


Figure (1): (a) HPLC chromatograms of rosemary extract, (b) Chemical structures of major bioactive phenolic compounds in rosemary leaves extract.

Table (1): A comprehensive list of the detected phytochemical compounds in the rosemary leaf extract by HPLC analysis.

No.	Detected Compound	Classification	Retion time (RT)	Molecular weight	Molecular formula	Area %
1	Apigenin	Flavonoid	11.57	270.24	C ₁₅ H ₁₀ O ₅	1.23
2	p-Anisic acid	Organic acid	12.73	167.16	C ₈ H ₈ O ₃	0.65
3	Hesperetin 7-O-rutinoside	Flavonoid	13.00	610.60	C ₂₈ H ₃₄ O ₁₅	2.55
4	Caffeic acid	Phenolic compound	13.50	180.16	C ₉ H ₈ O ₄	1.88
5	Teachtchrysin	Flavonoid	14.50	268.26	C ₁₆ H ₁₂ O ₄	0.92
6	Isorhamnetin	Flavonoid	19.00	316.26	C ₁₆ H ₁₂ O ₇	1.59
7	Vanillic acid	Organic acid	19.76	168.15	C ₈ H ₈ O ₄	0.64
8	1,2-Benzenediol	Organic acid	21.43	110.111	C ₆ H ₆ O	0.64
9	Methyl eugenol	Phenylpropanoid	22.00	178.23	C ₁₁ H ₁₄ O ₂	15.15
10	P-coumaric acid	Organic acid	23.76	164.16	C ₉ H ₈ O ₃	2.57
11	Ferulic acid	Organic acid	24.50	194.18	C ₁₀ H ₁₀ O ₄	3.14
12	Trans-Isoeugenol	Estragole	26.13	164.201	C ₁₀ H ₁₂ O ₂	1.69
13	3-hydroxybenzoic acid	Organic acid	29.82	138.12	C ₇ H ₆ O ₃	1.23
14	Estragole	Phenyl propanoid	30.27	148.20	C ₁₀ H ₁₂ O	0.62
15	Coumarin	Benzopyrone	31.81	146.143	C ₉ H ₆ O ₂	1.91
16	Gallic acid	Phenolic acid	32.79	170.12	C ₇ H ₆ O ₅	10.04
17	Carnosic acid	Phenolic diterpene	33.41	32.42 3	C ₂₀ H ₂₈ O ₄	13.81
18	Luteolin	Flavones	33.79	286.24	C ₁₅ H ₁₀ O ₆	2.13
19	Rutin	Flavonoid	34.00	610.50	C ₂₇ H ₃₀ O ₁₆	2.58
20	Rosmanol	Phenolic compound	34.15	346.40	C ₂₀ H ₂₆ O ₅	8.16
21	Protocatechuic acid	Phenolic compound	34.78	154.12	C ₇ H ₆ O ₄	0.63
22	Hispidulin 7-glucoside	Flavonoids	35.00	462.4	C ₂₂ H ₂₂ O ₁₁	5.64
23	Gentisic acid	Organic acid	35.62	154.12	C ₇ H ₆ O ₄	0.61
24	Rosmarinic acid	Organic acid	37.00	360.318	C ₁₈ H ₁₆ O ₈	9.42
25	Quercetin	flavonoid	40.24	302.23	C ₁₅ H ₁₀ O ₇	3.45
26	p-hydroxybenzaldehyde	Organic acid	40.76	122.12	C ₇ H ₆ O ₂	1.70

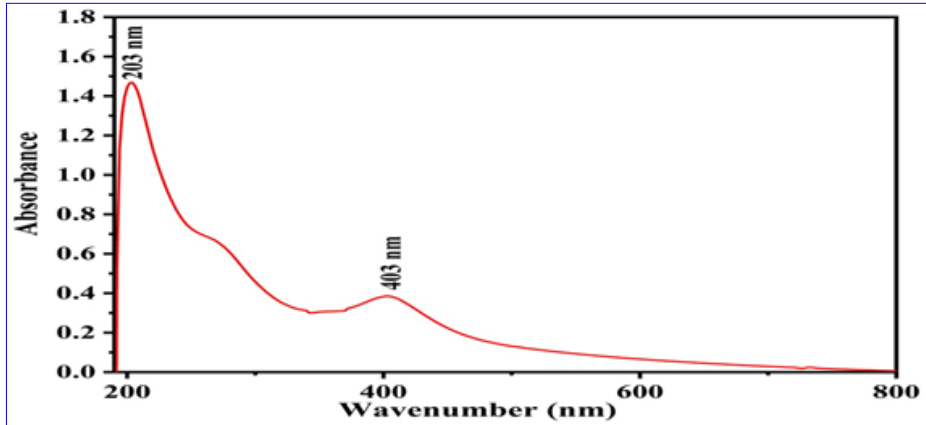


Figure (2): UV-Vis absorption spectra of AgNPs synthesized by aqueous rosemary leaves extracts.

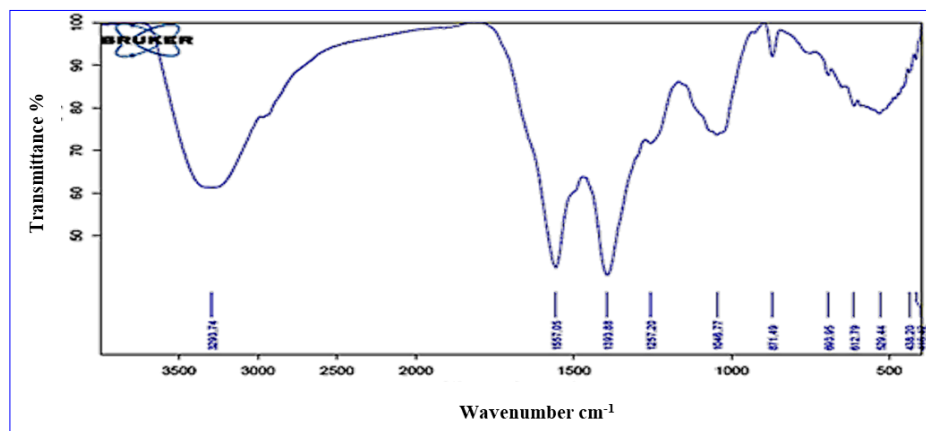


Figure (3): Fourier-transform infrared (FTIR) spectrum of Ag NPs rosemary leaves extract.

Table 2: Mortality and time-frequency of *P. gossypiella* and *E. insulana* treated with different concentrations of RNPs.

Tested Pests	Treatments	Tested Conc.	Observation time in day					Accumulative mortality in Number	Mortality (%)
			1	2	3	7	8-14 [†]		
<i>E. insulana</i>	Control	0	0.0	0.0	1.0	0.0	1.0	2.0	6.66
		40	17.0	18.0	13.0	11.0	1.0	60.0	100.00
	RNP	20	11.0	10.0	12.0	5.0	7.0	45.0	75.00
		10	7.0	5.0	8.0	11.0	2.0	33.0	55.00
		5	2.0	0.0	5.0	3.0	0.0	10.0	16.66
<i>P. gossypiella</i>	Control	0	0.0	0.0	0.0	2.0	2.0	4.0	6.66
		40	15.0	13.0	15.0	8.0	7.0	58.0	96.66
	RNPs	20	6.0	10.0	13.0	9.0	5.0	43.0	71.66
		10	4.0	6.0	9.0	8.0	1.0	28.0	46.66
		5	0.0	5.0	9.0	6.0	0.0	20.0	33.33

[†] Data recorded for the period 8-14 days. 60 newly hatched larvae for each concentration were used; RNPs Rosemary Nano Particles.

Table (3): Toxicological assessment of RNPs against newly hatched larvae of *E. insulana* and *P. gossypiella* under laboratory conditions, at three levels of lethal concentration values (LC₂₅, LC₅₀, and LC₉₀).

Treatment	Tested Pests	Lethal conc. values with 95% Confidence limits			
		25	50	90	Slope ± SE
RNP	<i>E. insulana</i>	8.25	16.75	64.39	2.1921 ± 0.2205
	<i>P. gossypiella</i>	6.38	18.65	143.29	1.4475 ± 0.2012

of *E. insulana* and *P. gossypiella* treated with the LC₅₀ levels of RNPs specifically exhibited increased malformation and mortality rates, represented in percentage, in both stages compared to the untreated larvae. The recorded weights of *E. insulana* were 0.0236 ± 0.001 mg and 0.0236 ± 0.003 mg for larva and pupa, respectively, compared to control which recorded 0.0409 ± 0.001 and 0.0354 ± 0.003 mg, respectively. For *P. gossypiella*, the weights were 0.029 ± 0.001 mg and 0.0019 ± 0.001 mg, for larvae and pupa, respectively, as opposed to 0.0380 ± 0.003 mg and 0.0250 ± 0.002 mg, respectively, in the untreated control group. In addition, *P. gossypiella* larvae and pupae were found to be deformed in 11.6 and 9.0% of treated individuals, respectively, as opposed to 2.1 and 1.6% for untreated ones.

Biochemical responses

After exposure to the LC₅₀ concentration of RNPs, the levels of total protein, lipid, and carbohydrate, along with the activity of invertase, trehalase, and proteinase supernatant were simultaneously evaluated in the larvae of *E. insulana* and *P. gossypiella* seven days post-treatment. Throughout the developmental stages of both insects, proteins, carbohydrates, and lipids were measured since they play essential roles as modified co- or post-translationally modified molecules within the insect's physiology. These components are considered as critical for the growth development and accomplishment of numerous cellular and biochemical processes in insect biology. Mainly carbohydrates play a significant role since they may be used by insects' bodies to produce energy or covert to lipid

or proteins to complete their physical processes.

Data in Table (6) indicated a decrease in total protein and carbohydrate levels to 48.5 and 11.5 mg/g, respectively, for the *P. gossypiella* strain, as opposed to 114.0 and 29.5 mg/g in the untreated control. Meanwhile, the total lipid content increased to 12.0 mg/g compared to 7.5 mg/g in the untreated control.

In contrast, a general decrease was observed for the treated *E. insulana*, particularly in the total lipid content, which was halved to 2.3 mg/g compared to 4.1 mg/g in the untreated control.

Hydrolyzing Enzymes

The tabulated and recorded data in Table (6) also showed a significant difference in the activity of the proteinase enzyme and the carbohydrate hydrolyzing enzymes (invertase and trehalase) in the supernatant of the homogenates treated larvae compared to that obtained with the untreated larvae. There was reduction of invertase activity in treated larvae (20.1 and 116.33 μ g glucose/g body weight/min) compared to control (30.2 and 129.0 μ g glucose/g body weight/min), also, trehalase activity decreased in treated larvae (101.0 and 98.0 μ g glucose/g body weight/min) compared to control (154 and 134 μ g glucose/g body weight/min) for the two pests *E. insulana* and *P. gossypiella*, respectively.

Invertase enzyme

Regarding invertase and trehalase enzymes, treatment throughout the test periods caused a reduction in activity in both insects compared to the control (Table 6). *P. gossypiella* and *E. insulana* larvae fed on LC₅₀ of RNPs compounds, tended to exhibit a high reduction in

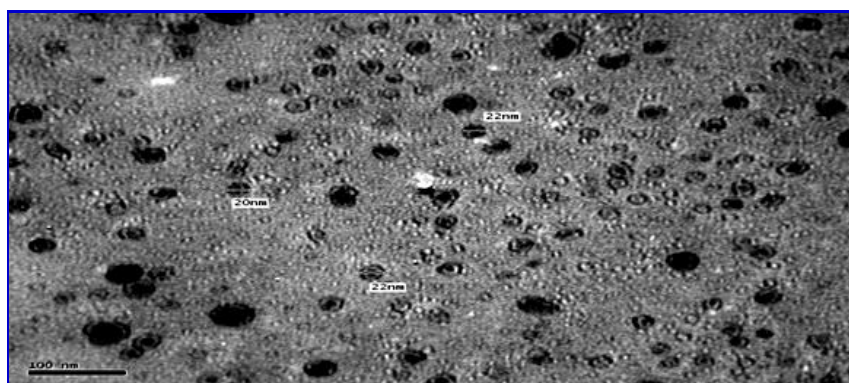


Figure (4): TEM images of synthesized AgNPs showing their size that ranged from 20-22nm. Bar, 100 nm.

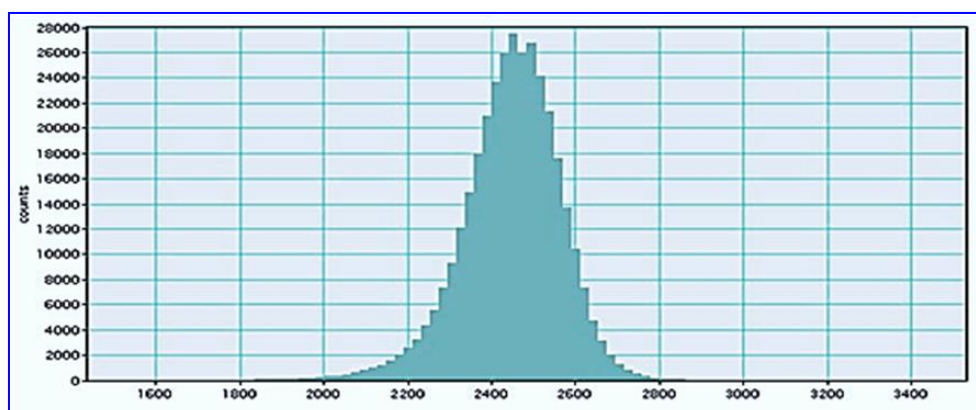


Figure (4): Histogram of the size distribution of AgNPs - rosemary leaves extract based on TEM image analysis.

Table (4): Impact of LC₅₀ of RNPs on developmental times of *E. insulana* and *P. gossypiella* under laboratory conditions

Tested Pests	Treatment	RNPs Conc. (%)	Larval time in days		Pupal time in days		Pupation%	Total immature duration (days)	Increase in duration (days)
			Duration	Increase in duration	Duration	Increase in duration			
<i>E. insulana</i>	Control	0.0	16.50 ^{bc}	0.0	7.80 ^b	0.0	83.0	24.30 ^b	0.0
	RNP	16.757	23.30 ^a	6.8	11.60 ^a	3.8	96.0	34.90 ^a	10.6
<i>P. gossypiella</i>	Control	0.0	14.60 ^c	0.0	7.30 ^b	0.0	100.0	21.90 ^b	0.0
	RNP	18.655	21.53 ^{ab}	6.93	12.30 ^a	5.0	100.0	33.83 ^a	11.93
	LSD		5.402	-	2.075	---	---	4.621	---
	F		6.1355	---	16.239	---	---	21.6392	---
	p [†]		0.018	---	0.0009	---	---	0.0003	---

[†]Means with different superscript letters, per column, are significantly different at $p \leq 0.05$ / $p \leq 0.01$.

Table (5): Impact of LC₅₀ on reduction % in weight and malformed of *P. gossypiella* and *E. insulana* larvae after treatment with LC₅₀ of nano particles under laboratory conditions

Pest	Treatment	Conc. (%)	Larvae		Pupae		Malformed Larvae%	Malformed Pupae%
			Weight (g)	Reduction %	Weight (g)	Reduction %		
<i>E. insulana</i>	Control	0	0.0409±0.001 ^a	0	0.0354±0.003 ^a	0.0	3.3	2.6
	RNP	16.757	0.0236±0.001 ^c	-42.3	0.0236±0.003 ^b	-33.33	9.6	12.6
<i>P. gossypiella</i>	Control	0	0.0380±0.003 ^a	0	0.0250±0.002 ^b	0	2.3	1.6
	RNP	18.655	0.0290±0.001 ^b	-23.68	0.0019±0.002 ^c	-92.4	11.6	9
	LSD		0.00518	---	0.00799	---	---	---
	F		31.9232	---	32.998	---	---	---
	p [†]		0.0001	---	0.0001	---	---	---

[†]Means with different superscript letters, per column, are significantly different at $p \leq 0.01$.

invertase enzyme activity. Compared to 30.0 and 129.0 in the control, invertase activity was 20.0 and 116.0 for the two pests, respectively.

Trehalase enzyme

The trehalase enzyme activity in treated *P. gossypiella* and *E. insulana* larvae was typically lower compared to controls, according to data shown in Table (6). It was respectively 101.0 and 98.0 µg glucose/g body weight/min, as opposed to 154.0 and 134.0 µg glucose/g body weight/min in untreated check.

Proteinase enzyme

Data in Table (6) illustrated the LC₅₀ treatment of RNPs against *P. gossypiella* and *E. insulana* neonates led to a significant increase in proteinase activity (84.0 and 153.0), in contrast to the control check (60.0 and 71.0), respectively.

Histological studies

In these studies, newly hatched larvae of *E. insulana* and *P. gossypiella* were fed artificial diets containing the LC₅₀ of *R. officinalis* nanoparticles. Histological studies were then conducted on the cuticle and midgut

of treated larvae after 10 days of treatment, and compared to untreated larvae.

Untreated *E. insulana* and *P. gossypiella* larva exhibited normal histological structure under light microscopy, with spines and a corrugated cuticle surface overlaying the underlying epidermis (Figs. 6 A and B). Examination of longitudinal sections of untreated *E. insulana* and *P. gossypiella* larvae indicated a typical histological structure of the cuticle layers, with clearly distinguished epidermis, buds and spines (Figs. 7 and 8). However, LD₅₀ of RNPs, showed deformation and necrosis with loss in spines protruded spines in the examined structures (Fig. 9 A and B).

Conversely, histological sections of the gut regions of both insects also revealed typical glandular and cellular architecture, with tightly packed columnar epithelial cells resting on a basement membrane and keeping the gut lumen isolated from it by an intact peritrophic membrane. Therefore, the untreated larvae showed no histopathological changes (Figs. 10A and B).

Table (6): Impact of LC₅₀ of tested nanoparticles on some biochemical aspects of *P. gossypiella* and *E. insulana* under laboratory conditions

Pest Tested	Treatment	Measured parameters (mg/g)			Invertase	Trehalase	Proteinase
		Protein	Lipid	Carbohydrate			
<i>E. insulana</i>	Control	114.0±6.5 ^a	7.5±0.3 ^b	29.5±0.96 ^a	30.2±2.20 ^c	154.0±2.50 ^a	60.0±4.60 ^{bc}
	RNP	48.5±6.5 ^b	12.0±3.5 ^a	11.5±2.40 ^c	20.1±1.30 ^c	101±7.10 ^c	84.0±5.30 ^b
<i>P. gossypiella</i>	Control	73.7±4.41 ^b	4.11±0.9 ^c	15.34±1.20 ^b	129.0±6.93 ^a	134.0±10.9 ^b	71.0±5.90 ^{bc}
	RNP	49.6±4.9 ^b	2.3 ^d	6.4±0.11 ^d	116.33±22.58 ^b	98.0±6.90 ^c	153±11.60 ^a
	LSD	30.696	1.779	3.79	12.479	8.0435	15.151
	F	10.609	61.585	61.909	219.45	119.71	81.081
	p	0.0037	0.000	0.000	0.000	0.000	0.000

[†]Means with different superscript letters, per column, are significantly different at $p \leq 0.01$.

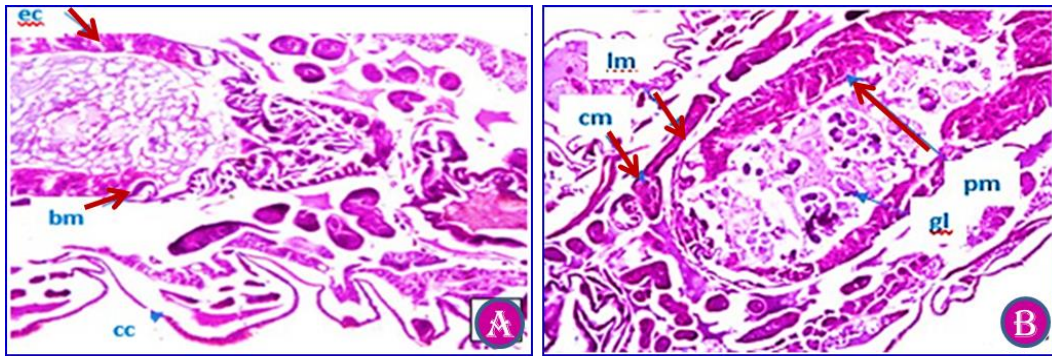


Figure (6): L.S. of *P. gossypiella* (A), *E. insulana* (B) untreated larva (X16) showing cuticle layers (cl), Longitudinal muscle layer (lm), Circular muscle layer (cm) epithelial columnar cell (ec), basement membrane (bm), Peritrophic membrane (pm) and the inner gut lumen (gl).

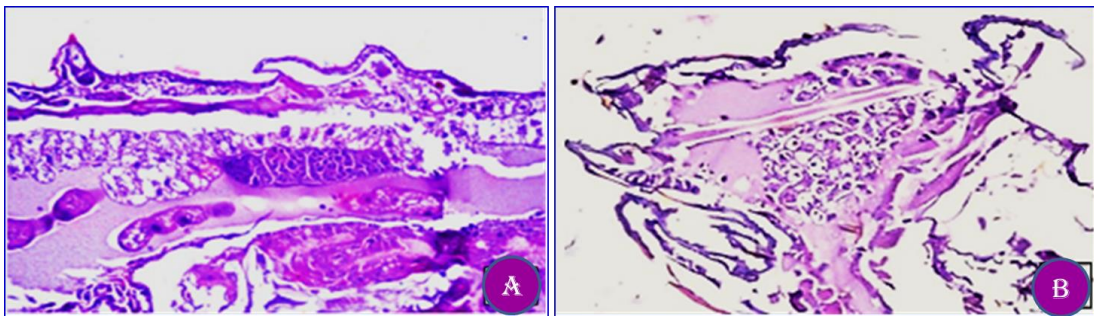


Figure (7): L.S. of *E. insulana* (A), *P. gossypiella* (B) larva treated as a neonate with LC₅₀ of *Rosmarinus officinalis* L (X16) showing a general view of cuticle layers with the inner gut.

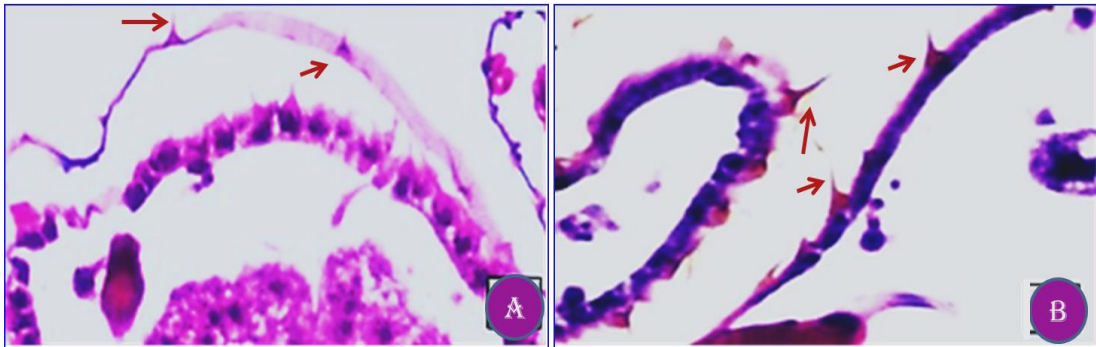


Figure (8): Normal cuticle layer of *E. insulana* (A), *P. gossypiella* (B) untreated larva with normal structure of buds and spines.

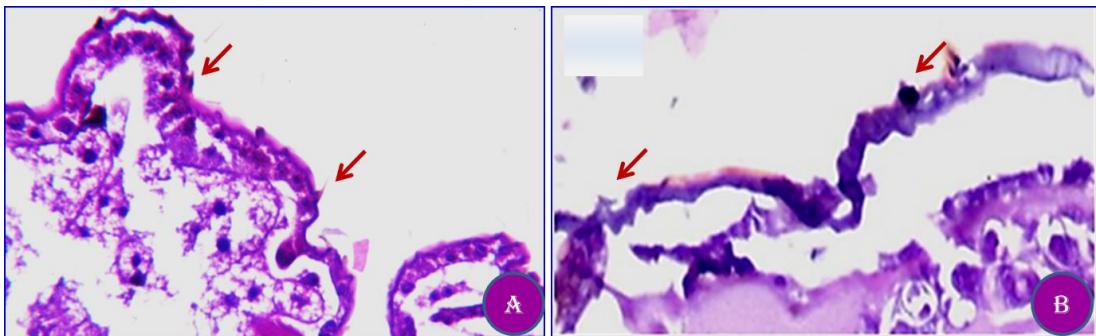


Figure (9): Cuticle layer of *E. insulana* (A) and *P. gossypiella*, (B) *R. officinalis* L. treated larvae showed a loss in spines, the outline was fold and necrosis with fusion and compactness of the blurred cuticle layers.

In parallel, magnification of the mid-gut epithelial layer of *E. insulana* (Fig 11A) and *P. gossypiella* (Fig. 11B), untreated larva indicating the normal structure of the columnar cells with a large central nucleus that rests on the basement membrane and is lined by the peritrophic membrane.

On the other hand, the microscopic examination revealed some histological alternations for both insects at the level of the gut region as a result of *R. officinalis* nanoparticles (RNPs) treatment showed a loss of the compact appearance and vacillations of epithelial cells with exofliation of the columnar epithelial layer and disrupted peritrophic membrane (Figs. 12A and B) for *E. insulana*, and *P. gossypiella*, respectively.

In the meantime, treated insects displayed disarray and vacuolization of the columnar cells in the mid-gut epithelial layer of *E. insulana* (Fig. 13 A) and *P. gossypiella* (Fig. 13 B). This observation indicates significant damage to the mid-gut region. Additionally, the peritrophic membrane completely degraded, the epithelial cells at the boundaries of the epithelial cells also were destroyed, and the basement membrane detached in certain areas, causing disruption and fusion in the columnar epithelium cells and necrotic epithelium with basal nuclei (Fig. 13 A and B).

In general, the histopathological alterations listed below were noticed: a, disarray and Vacuolization where the mid-gut epithelial layer's columnar cells displayed vacuoles in addition to being elongated and disorderly. b, peritrophic membrane degradation: in which the peritrophic membrane, that ordinarily shields the mid-gut epithelium, was totally destroyed. c, destroying epithelial cell boundaries in which the mid-gut epithelium's boundary epithelial cells were damaged. In addition, basement membrane detachment was also observed. This basement membrane that gives the epithelium layer structural support, was separated in some places. The columnar epithelial cells also were disrupted and fused as a result of these histological alterations, and necrotic epithelium with basal nuclei was present.

DISCUSSION

Substituting chemical insecticides with naturally derived plant extracts is a sustainable approach to pest management. This shift mitigates the adverse effects of environmental contamination and safeguards human well-being (Pathak *et al.*, 2022; Sabry *et al.*, 2023). Essential oils in rosemary possess potent insecticidal properties due to bioactive compound as cineole, camphor, and borneol, exhibit strong pesticidal effects against a wide range of insects. When applied appropriately, rosemary-based formulations can effectively deter, repel, or even kill target pests (Micić *et al.*, 2021).

The first sign of silver nanoparticle synthesis was the observable change in the solution's color, evolving from a clear, pale yellow to a distinctive brown tint (Abdel-Aziz, 2019). This shift in hue is a result of the activation of free electrons, leading to the formation of absorption bands via surface plasmon resonance (SPR).

This phenomenon occurs due to the synchronized oscillation of electrons in harmony with light waves, as elucidated by Ammar and Abd-ElAzeem, 2021 in their study. The UV-visible spectra displayed highest absorption levels within the range of 300 to 700 nm. This phenomenon was linked to the activation of electrons in the conductive band located near the surface of the nanoparticles. Correspondingly, in a study conducted by Ammar and Abd-ElAzeem (2021), they observed distinct peaks at 460 and 414 nm, which indicated the production of CuNPs. Moreover, nano-form plant extracts can be precisely engineered to release their active compounds in a controlled manner, ensuring sustained efficacy over an extended period (Wang *et al.*, 2022)

In essence, our study evaluated the influence of eco-friendly RNPs derived from natural sources on the larval stage of *P. gossypiella* and *E. insulana*. It was discovered that using nano-formulated plant extracts for pest control is more effective than non-nano forms. This is because reducing particle size to the nanoscale increases the surface area for pest interaction, enhancing bioavailability and uptake Grillo *et al.*, 2016. Additionally, the smallest silver nanoparticles in our study (20 to 22 nm) allow them to swiftly enter cells, thereby disrupting key physiological functions of the insect while Stadler *et al.* (2010) recorded that the size of synthetic AgNPs between 35 and 60 nm exhibited the highest efficacy in their crude aqueous against *C. quinquefasciatus* ($LC_{50} = 27.49$ & 4.56 mg/L; $LC_{90} = 70.38$ & 13.14 mg/L); and against *A. subpictus* ($LC_{50} = 27.85$ & 5.14 mg/L; $LC_{90} = 71.45$ & 25.68 mg/L). Aside from that, Rajakumar and Abdul Rahuman, 2011; Zahir *et al.* (2012) discovered the potential of *Eclipta prostrata* leaf extracts which employed as an optimal environmentally friendly method for the control of *Sitophilus oryzae*. The aqueous extract, AgNO₃ solution and synthesized AgNPs had LD₅₀ values of 213.32, 247.90, and 44.69 mg/kg⁻¹, respectively, and LD₉₀ values of 1638.08, 2675.13, and 168.28 mg/kg⁻¹.

The larvicidal activity of synthesized silver nanoparticles recorded using the leaf extract of *Tinospora cordifolia* (Jayaseelan *et al.*, 2011) that caused high percent mortality against *Pediculus humanus* and fourth instar larvae of *Anopheles subpictus*. Rajakumar and Abdul Rahuman, 2011, along with Zahir *et al.*, 2012, identified the potential of silver nano particles *Eclipta prostrata* leaf extracts as an effective and environmentally friendly method for controlling *Sitophilus oryzae*. The LD₅₀ values for AgNPs were 213.32, 247.90, and 44.69 mg/kg⁻¹, respectively. The LD₉₀ values were 1638.08, 2675.13, and 168.28 mg/kg⁻¹. Furthermore, Jayaseelan *et al.* 2011 synthesized silver nanoparticles using *Tinospora cordifolia* leaf extract exhibited high mortality rates against *Pediculus humanus* and fourth instar larvae of *Anopheles subpictus*. Nanoparticles present possibilities for more efficient and effective control of pests (Khot, 2012).

In HPLC analysis, certain compounds in rosemary

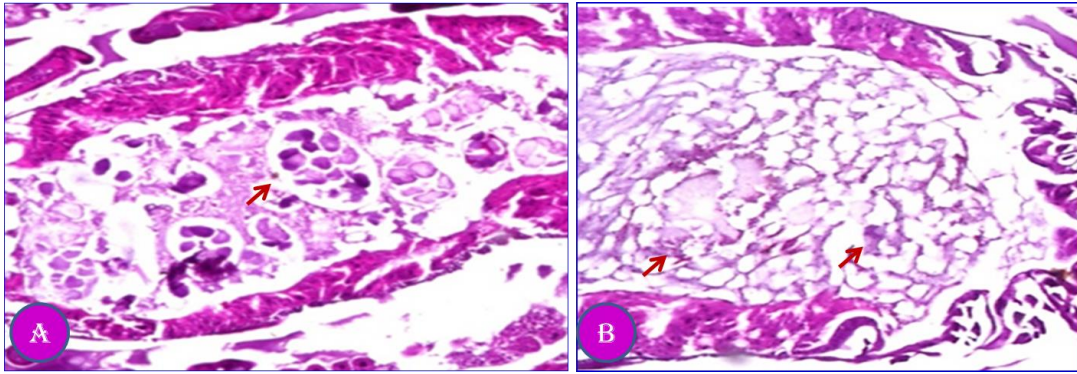


Figure (10): Light microscopic examination shows the midgut of normal, *E. insulana* (A), *P. gossypiella* (B) untreated larva consists of a single cellular epithelial layer with a large granular nucleus resting upon a basement membrane which is lined with a thin peritrophic membrane, surrounding the food mass. Magnification =16X.

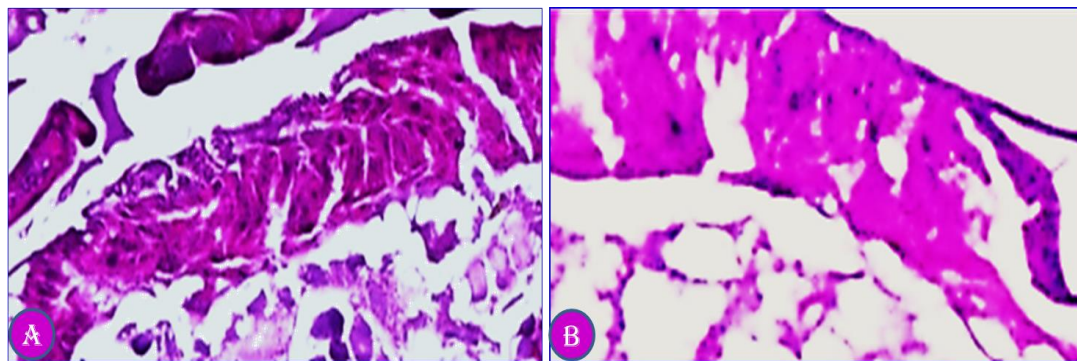


Figure (11): Magnification of the mid-gut epithelial layer of *E. insulana* (A) and *P. gossypiella* (B), untreated larva indicating the normal structure of the columnar cells with its central large nucleus, resting on the basement membrane and lining with the peritrophic membrane. Magnification = 40X.

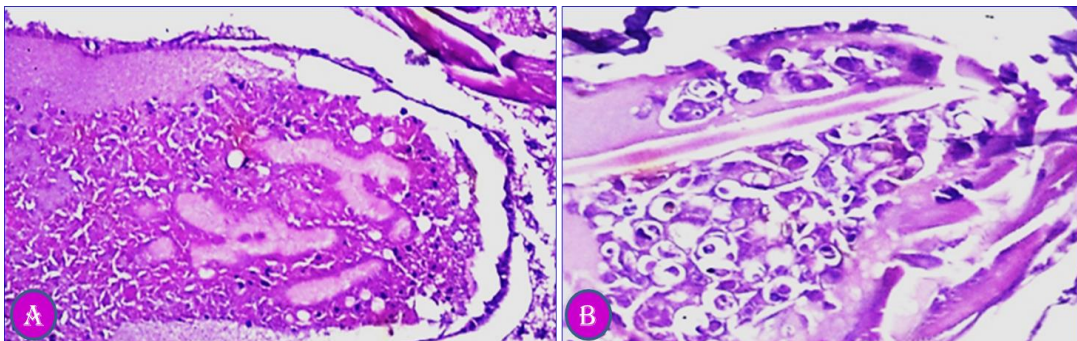


Figure (12): L.S. of treated larva of *E. insulana* (A), and *P. gossypiella* (B), showing loss of the compact appearance and vacillations of epithelial cells with exfoliation of the columnar epithelial layer and disrupted peritrophic membrane. Magnification = 40X.

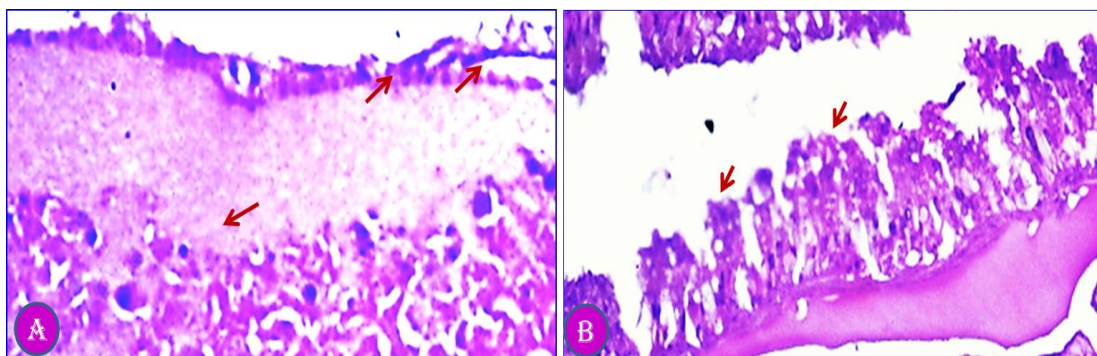


Figure (13): Magnification of the midgut epithelial layer of *E. insulana* (A) and *P. gossypiella* (B) treated larvae shows disorganization and vacuolization of the columnar cells, as well as complete deterioration of the peritrophic membrane. Additionally, destruction of the epithelial cells and extensive damage to the boundaries of epithelial cells lead to disruption and fusion in the columnar epithelium cells, resulting in necrotic epithelium with basal nuclei and detachment of the basement membrane in some regions. Magnification = 40X.

extracts can interfere with the feeding and reproductive processes of insects. This disrupts their life cycle and population growth, contributing to effective pest control (Nieto *et al.*, 2018). Intermediate larvae-pupal or pupal-adult was the most recorded morphological deformation. Results obviously indicated that nano extract is highly effective with toxic potential against the two larval and pupal stages of both tested insects. This impediment response may be related to several toxic mechanisms of rosemary nanoparticles that have been documented in the toxicity table. This finding is consistent with (Ammar and Abd-ElAzeem, 2021) which demonstrated that nano copper was highly toxic and inhibits the growth of *E. insulana*.

According to our findings, (RNPs) had a far greater mortality effect on *E. insulana* larvae than on *P. gossypiella* larvae. This increase in mortality was caused by more exposure of *E. insulana*'s cuticle to the compound than *P. gossypiella*'s (Rouhani *et al.*, 2008). Debnath *et al.* (2011) applied Silica NPs can considerably increase the mortality effect of NPs as the period after application increases. Ultimately, the present study's observations of changes in the activities of carbohydrate hydrolyzing enzymes and/or proteinase enzymes may have an impact on growth, weight, and metabolic process, and may therefore help to explain the cause of mortalities and malformations for different stages in both insects as previously demonstrated (Ammar and Abd El-Azeem, 2021).

CONCLUSION

This study demonstrated the promising potential of silver nanoparticles derived from *Rosmarinus officinalis* leaves as an effective and eco-friendly alternative to traditional chemical pesticides. The AgNPs were successfully synthesized and characterized, showing toxicity against the common agricultural pests *Earias insulana* and *Pectinophora gossypiella*. The nanoparticles exhibited LC₅₀ values in the mid-teen percentage range, indicating good potency against these insects. Treatment with the Rosemary-AgNPs caused adverse effects such as prolonged development, disrupted biochemistry, and histological irregularities in the cuticle and midgut tissues. These results emphasize the viability of utilizing nature-derived solutions like plant-mediated nanoparticles for pest control. Further research optimizing nanoparticle production and application methods could help advance the use of these eco-friendly alternatives to conventional insecticides. Generally, the study provides promising evidence for silver nanoparticles as a sustainable pest management tool addressing growing environmental concerns around chemical pesticide usage.

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استخدام جزيئات الروزماري النانوية صديقة البيئة ضد اثنين من ديدان لوز القطن: دراسة الآثار البيولوجية والبيوكيميائية والهستولوجية

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الملخص العربي

هناك احتياجات ضد الاستخدام الواسع للمبيدات الحشرية. هدفت هذه البحوث إلى إلقاء الضوء على بديل صديق للبيئة يستخدم جسيمات الفضة النانوية المستمدة من نبات أكليل الجبل *Rosmarinus officinalis* وتم اختبارها ضد ديدان اللوز القرنفلية والشوكية *Earias* و *Pectinophora gossypiella* و *insulana* كانت طيفية UV-VIS لحزمة امتصاص جزيئات الفضة نانوية للروزماري عند حوالي 403 نانومترًا، وهو سمة مميزة للفضة، مما يشير إلى نجاح تخليق الجسيمات النانوية. تم التعرف على ستة وعشرين مركبًا من *Rosmarinus officinalis* من خلال تحليل HPLC. بالإضافة إلى ذلك، أظهرت صور الميكروسكوب الإلكتروني الشكل الكروي للجسيمات النانوية الفضية بقطر متوسط 20-22 نانومترًا. تم تحديد قيم LC_{50} لـ *P. gossypiella* و *E. insulana* على التوالي بنسبة 18.655 و 16.75%. علاوة على ذلك، أظهر التمديد الكبير في المراحل التطورية لكل من الحشرات، حيث استغرقت المراحل الإجمالية 33.83 و 34.9 يومًا لـ *P. gossypiella* و *E. insulana* على التوالي مقارنة بالمجموعة غير المعالجة 21.9 و 24.3 يومًا. أظهر التحليل الكيميائي الحيوي انخفاضًا ملحوظًا في مستويات البروتين والكربوهيدرات، حيث انخفضت إلى 48.5 و 11.5 ملغ جلوكوز/جم، على التوالي، مقابل 114.0 و 29.5 في الغير معالجة. على الجانب الآخر، هناك زيادة ملحوظة في محتوى الدهون الكلية، حيث بلغت 2.0 ملغ/جم في سلالة *P. gossypiella*، مقارنة بـ 7.5 في المجموعة غير المعالجة. ومع ذلك، في *E. insulana*، هناك انخفاض كبير إلى 3.2 ملغ/جم، مقارنة بـ 4.1 ملغ/جم في المجموعة غير المعالجة. أيضًا، أدت المعالجات إلى تغيرات نسبية في القشرة والأمعاء المتوسطة في كلا الحشرتين مقارنة بالغير المعالجة.