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Assessment of the Quantitative and Qualitative Residual Effect in Wax Frames Treated with Nano-Neem Extract

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Abstract: The study aims to investigate the residual effect of silver nanoparticles synthesized from neem (Azadirachta indica), by evaluating the ability of honey bees to remove these compounds through their hygienic behavior in wax frames treated with the nano-extract. The application of silver nanoparticles in combination with neem seed extract enhances biological efficacy while reducing environmental impact, thus opening prospects for developing safe and sustainable control strategies. Quantitative and qualitative identification of the nano-compounds were performed by using several analytical instruments, including: Fourier Transform Infrared Spectroscopy (FT-IR) to detect functional groups, Gas Chromatography–Mass Spectrometry (GC-MS) to identify bioactive nano-compounds, and Atomic Absorption Spectrophotometer (AAS) to determine the quantitative concentration of silver nanoparticles. The results were showed that the concentration of silver in the neem nano-extract was 136.7 ppm before exposure to cleaning, while it significantly decreased in the wax frames treated with the nano-extract to 0.3 ppm after being cleaned by worker bees. In our conclusion is considered low and safe, as it falls within acceptable limits for human health

Keywords: Neem Plant, Silver Nano-Extract, GC-MS, FT-IR, Atomic Absorption

Introduction

Neem (Azadirachta indica) is an important medicinal plant that grows in tropical and subtropical regions, widely recognized for its significance in traditional medicine and agriculture. It contains a large variety of bioactive compounds that provide multiple therapeutic and biological properties, such as: Limonoids: the most well-known are Azadirachtin, Nimbin, Salannin, and Nimbolide, which are responsible for insecticidal and pesticidal effects. Flavonoids and Tannins: act as antioxidants and anti-inflammatory agents. Glycosides and Steroids: play roles in immune enhancement and other biological activities. Other compounds such as Nimbidin, Gedunin, and Mahmoodin, which contribute to neem's antimicrobial, anti-inflammatory, and pesticidal properties.

These constituents make neem a plant of high pharmaceutical and agricultural value, as well as an alternative to reduce dependence on conventional chemical pesticides. Additionally, In many parts of the world, yellow maize is a major crop for both food and industry. In terms of planted areas, it comes in third place globally, after rice and wheat. per

hectare, based on Ministry of Agriculture figures. With advancements in nanotechnology, neem extract can be utilized in the green synthesis of silver nanoparticles (AgNPs). The extract acts as both a reducing and stabilizing agent, leading to the production of stable nanoparticles with strong biological properties. Silver nanoparticles synthesized using neem have shown high antimicrobial and insecticidal activity, in addition to their ability to interact with pest biological structures and reduce resistance development. Integrating neem extract with silver nanoparticles provides a promising approach in protecting bee products such as wax frames, by reducing contamination from pests and microbes while ensuring product safety. Accordingly, this study aims to assess the quantitative and qualitative residual effect of silver nanoparticles synthesized from neem extract in wax frames, through analyzing residual contamination levels and determining their chemical characteristics.

Research Method

Extraction Process of Neem Nano-Compound

The neem nano-extract is used as a reducing agent and a capping agent to transform silver ions (Ag^+) into metallic silver nanoparticles (Ag^0). Distilled water or a 9:1 water:ethanol mixture is used to maximize the extraction of phenolic compounds. The plant material (leaves/seeds/roots) is washed thoroughly with distilled water to remove dust, then cut into small pieces or coarsely ground (for seeds). An amount of 5–10 g of seeds is added to 100 mL of water, followed by cold soaking for 1–24 hours with shaking to prevent the loss of heat-sensitive compounds. Alternatively, 90% water + 10% ethanol can be used to extract less water-soluble compounds (especially from seeds). Afterwards, a 1 mM silver nitrate ($AgNO_3$) solution is prepared as follows: dissolve 0.01699 g of $AgNO_3$ in 100 mL of distilled water and store in the dark. To synthesize AgNPs, 10 mL of neem extract is mixed with 90 mL of the $AgNO_3$ solution (1 mM), making a total of 100 mL. The mixture is stirred on a magnetic stirrer at room temperature or gently heated (30–60°C). The reaction time ranges between 20 minutes to 2 hours, depending on activity. A visible color change of the solution to yellow/brown indicates the formation of AgNPs. The nanoparticles are then purified by centrifugation (10,000–15,000 rpm, 15–30 min), washed twice with distilled water, and redispersed.

Effect of Neem Nano-Extract on Honey Bees and Their Hygienic Behavior Under Apiary Conditions

Before starting the main experiment to evaluate the effectiveness of neem nano-extract against the greater wax moth, preliminary tests were conducted to examine the hygienic behaviour of bees and the effect of neem nano-extract on honey bees, as well as its residual impact on wax and stored honey in frames. New wax foundations were used and placed inside colonies of equal and high density. After 48 hours, the wax foundations were removed. during which bees had started to build natural wax. These frames were then treated with neem nano-extract at 15% concentration and returned to the colonies. Continuous monitoring was performed to observe bee acceptance of the treatment and their

hygienic behavior. The experiment included three replicates (one frame per replicate). For the control treatment, frames were left untreated. After 14 days, the frames were inspected to observe honey storage. then samples of wax and honey were collected for laboratory analyses to detect residual nano-materials.

Analysis Using Fourier Transform Infrared Spectroscopy (FT-IR)

One micro-litter was taken from each of the following three samples:

1. Neem seed nano-extract (15%) sprayed on wax cells, after which 5–10 g of wax comb (built by bees after 2 weeks) was collected and extracted using a Soxhlet apparatus with 70% solvent.
2. Raw neem seed extract sprayed on wax cells, then 5–10 g of wax comb was collected and extracted using Soxhlet.(%70)
3. Wax comb (5–10 g) produced by bees without any treatment (control), also extracted with Soxhlet.

The FT-IR analysis was performed at the Central Laboratory of the College of Agriculture and Forestry. A sufficient amount of each extract was placed on the measuring chamber (crystal window), and an infrared beam was directed through the sample. Each sample absorbed specific wavelengths according to its chemical structure and bonds. The results were recorded as spectra with peaks, where each peak corresponded to a particular compound.

Analysis Using Gas Chromatography–Mass Spectrometry (GC-MS)

The analysis was conducted by using a Shimadzu GC-MS QP210 Ultra system (Japan), equipped with a capillary column of type DB-5 (5% phenyl, 95% methyl polysiloxane), 30 m in length, 0.32 mm internal diameter, and 0.25 μm film thickness. The carrier gas was high-purity helium (%99.999) The thermal program was as follows: the oven temperature started at 40°C for 5 minutes, increased at 5°C/min to 150°C, and continued at 5°C/min until reaching 280°C, where it was held for 20 minutes. An AOC-Shimadzu autosampler (20i+s) was used, with injection performed in split mode at a ratio of 1:56. The injector temperature was 280°C, while the initial column temperature was 40°C. Each sample required approximately 3 minutes for analysis. The spectra of the separated compounds (peaks) were analyzed using GC-MS Solution software, and matched against the NIST 08 database for precise identification. The Soxhlet-extracted neem seed nano-extract (15%) mentioned above was sent to the University of Baghdad, College of Technology, where 1 mL of the extract was subjected to GC-MS analysis. Method for Measuring the Residual Effect of Samples (Neem Seed Nano-Extract, Wax Cells .5 Treated with Nano-Extract, and Untreated Wax Cells) Using Atomic Absorption Spectrophotometer (A.A.S.).

Estimation of Silver Element by Using Atomic Absorption

There are several methods for estimating silver; in this study, the following samples were analyzed:

1. One milliliter of neem seed nano-extract (15%) obtained using Soxhlet extraction.
2. Neem seed nano-extract sprayed on wax cells, from which 5–10 g of wax cells were collected and extracted using Soxhlet with 70% ethanol.
3. Wax comb produced by honey bees without any treatment (control), extracted using Soxhlet.

The most accurate and widely used method for determining silver is the Atomic Absorption Spectrophotometer (A.A.S.), which is considered the best technique for silver estimation. Using the wet digestion method, silver is quantified after the sample is digested to convert the element into a soluble form. Measurements are then performed with the atomic absorption spectrophotometer, with standard silver calibration solutions prepared in gradient concentrations. The two extracts (wax cells treated with neem nano-extract and untreated wax cells as control) were digested using the wet digestion method. Ten grams of each sample were incinerated in a furnace to obtain ash. Concentrated sulfuric acid (H_2SO_4) and perchloric acid ($HClO_4$) were then used: 0.5 g of the powdered sample was taken and 10 mL of concentrated sulfuric acid was added, left for 24 hours, after which drops of perchloric acid were added while heating on a hot plate. The digested extract was then quantitatively transferred into a 100 mL volumetric flask and diluted with distilled water up to the mark.

Result and Discussion

FT-IR Spectrum and Analysis of Active Compounds

Table 1. analyzing the results of the active compounds using FT-IR

Sample	Peaks (cm^{-1})	Functional Group / Possible Compound	Scientific Interpretation	Biological Role
Nano Neem Extract + Silver		Azadirachtin	Main neem compound	Insecticidal effect
		Nimbin & Nimbidin	Neem bitter compounds	Antibacterial and antifungal
		Polyphenols & Flavonoids	Natural polyphenolic compounds	Strong antioxidants
		Fatty acids & Terpenoids	Fatty acids and terpenoids	Support biological activity
Raw Neem Extract + Silver (FT-IR)	3383–3284	OH (alcohols, water)	Broad hydroxyl group peak	Presence of ethanol / microbial activity
	2977–2889	C–H (Alkyl groups)	Aliphatic carbon bonds	Energy source and active compounds
	2341	CO_2 (atmospheric absorption)	Weak peak from environment	Not biologically relevant
	1651	C=O or C=C	Ketones, aldehydes, aromatic compounds	Antimicrobial activity
	583–434	M–O (ZnO, TiO_2, Fe_2O_3)	Metal oxide nanoparticles	Antibacterial and antifungal, enhance activity

Sample	Peaks (cm^{-1})	Functional Group / Possible Compound	Scientific Interpretation	Biological Role
Beeswax (Control – untreated)	1646.46	C=O or C=C	Carboxylic acids, ketones, aldehydes, amides	Biologically active polar compounds
	1083.15 / 1043.41	C–O	Alcohols, ethers, esters	Aid solubility and microbial activity
	871.82	C–H (aromatic or alkenes)	Aromatic compounds (phenols)	Natural antioxidants
	457.68 / 429.98 / 417.20	M–O	Metal oxide nanoparticles (Fe–O, Zn–O, Al–O)	Enhance biological activity, antimicrobial

When analyzing the FT-IR spectrum, several characteristic peaks appear at specific wavelengths (cm^{-1}), each corresponding to certain bonds or functional groups. These allow the identification of bioactive compounds in the sample.

- Sample 1: Neem Seed Nano-Extract with Silver Nanoparticles (Soxhlet Extracted)

The FT-IR analysis showed that the neem nano-extract contains highly active compounds such as:

- Azadirachtin: a major insecticidal compound.
- Nimbin & Nimbidin: antimicrobial and antifungal agents.
- Polyphenols & Flavonoids: strong antioxidants.
- Fatty Acids & Terpenoids: organic compounds playing roles in bioactivity.

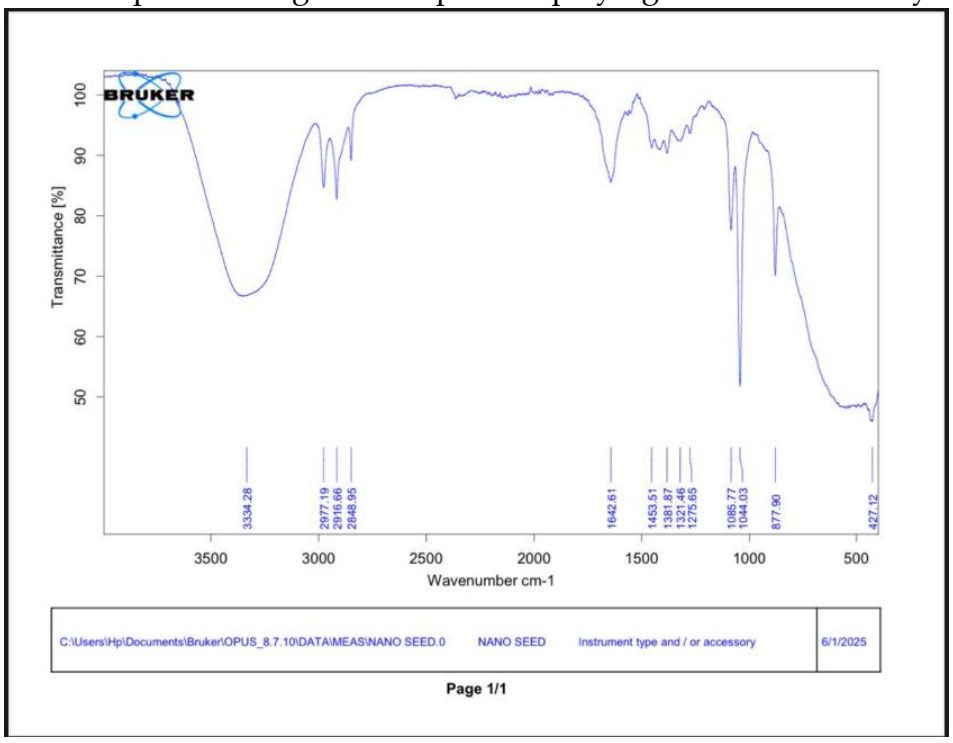


Figure 1. Neem Seed Nano-Extract Loaded with Silver

Sample 2: Raw Neem Seed Extract with Silver, Sprayed on Wax Cells and Extracted Using Soxhlet

1. Peaks at 3383 – 3284 cm^{-1}: A broad and deep peak, highly characteristic of the hydroxyl (OH) group.
   - Possible compounds: Alcohols such as ethanol (C_2H_5OH). Water or residual moisture may also be present.
   - The presence of this broad peak indicates that alcohol is present in a noticeable concentration.
2. Peaks at 2977 – 2889 cm^{-1}: Sharp, medium-intensity peaks, corresponding to aliphatic carbon–hydrogen (C–H) stretching.
   - Possible compounds: Ethanol, simple aliphatic compounds, or organic impurities containing carbon chains.
3. Peak at 2341 cm^{-1}: A relatively weak peak, most likely due to atmospheric carbon dioxide absorption during the analysis.
   - Possible compounds: Not a major compound in the sample, likely an impurity or environmental contamination.
4. Peak at 1651 cm^{-1}: A distinct and characteristic peak, indicating the presence of double bonds.
   - Possible compounds: Small amounts of aldehydes or ketones, or simple aromatic compounds.
   - This may result from ethanol degradation or nanoparticle additives.
5. Peaks from 583 to 434 cm^{-1}: Low-energy peaks that are highly significant in nanomaterial spectra.
   - Possible compounds: Nanoparticles containing metal oxides (such as ZnO, TiO_2, Fe_2O_3).
   - These nanometal oxides exhibit strong antibacterial and antifungal properties and act as catalysts to enhance the bioactivity of the extract. Their presence provides evidence of silver or other metals being integrated into the nano-structure of the extract.

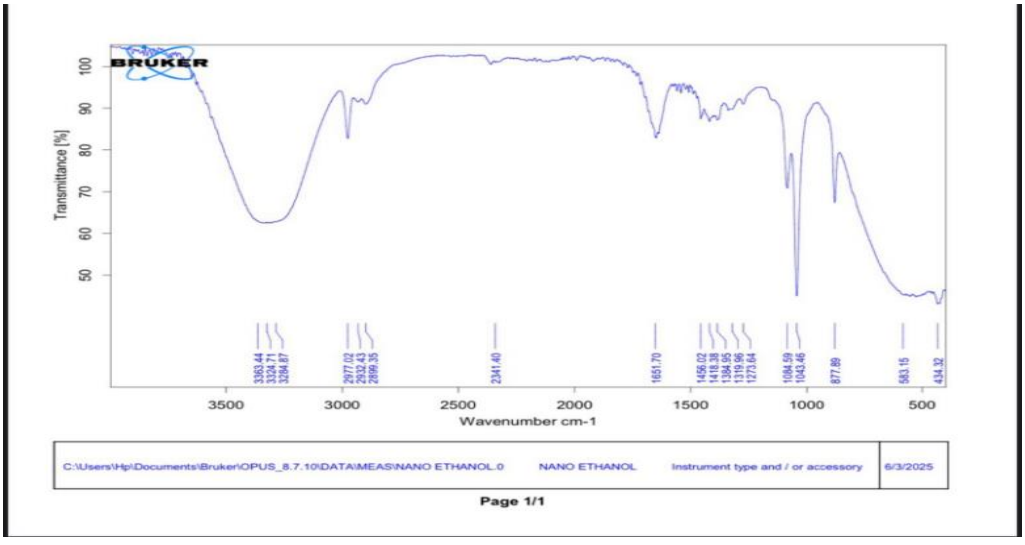


Figure 2. Raw Neem Seed Extract Sprayed on Wax Cells

Sample 3: Wax Produced by Honey Bees Without Any Treatment (Control), Extracted Using Soxhlet

1. Peak at 1646.46 cm^{-1}: Represents a carbonyl group (C=O) or a double bond (C=C) in alkenes, carboxylic acids, or amides.
   - Possible compounds: Carboxylic acids, ketones, aldehydes, amides.
   - These are biologically active polar compounds that support antimicrobial and antifungal effects.
2. Peaks at 1083.15 and 1043.41 cm^{-1}: Vibrations of C–O bonds in alcohols, ethers, or carboxylic acids.
   - Possible compounds: Alcohols, ethers, esters.
   - These polar compounds enhance solubility and contribute to antimicrobial and antifungal activity.
3. Peak at 871.82 cm^{-1}: Indicates C–H bonds in aromatic rings or alkenes.
   - Possible compounds: Aromatic compounds such as phenols, or compounds containing a benzene nucleus.
   - These act as natural antioxidants and contribute to biological activity.
4. Peaks at 457.68, 429.98, and 417.20 cm^{-1}: Vibrations of metal–oxygen bonds (such as Fe–O, Zn–O, Al–O), commonly found in nanomaterials or metal complexes.
   - Possible compounds: Metal oxide nanoparticles, neem-based nano-complexes, or plant extracts containing metals.
   - These metallic nanoparticles enhance bioactivity and increase antimicrobial potential.

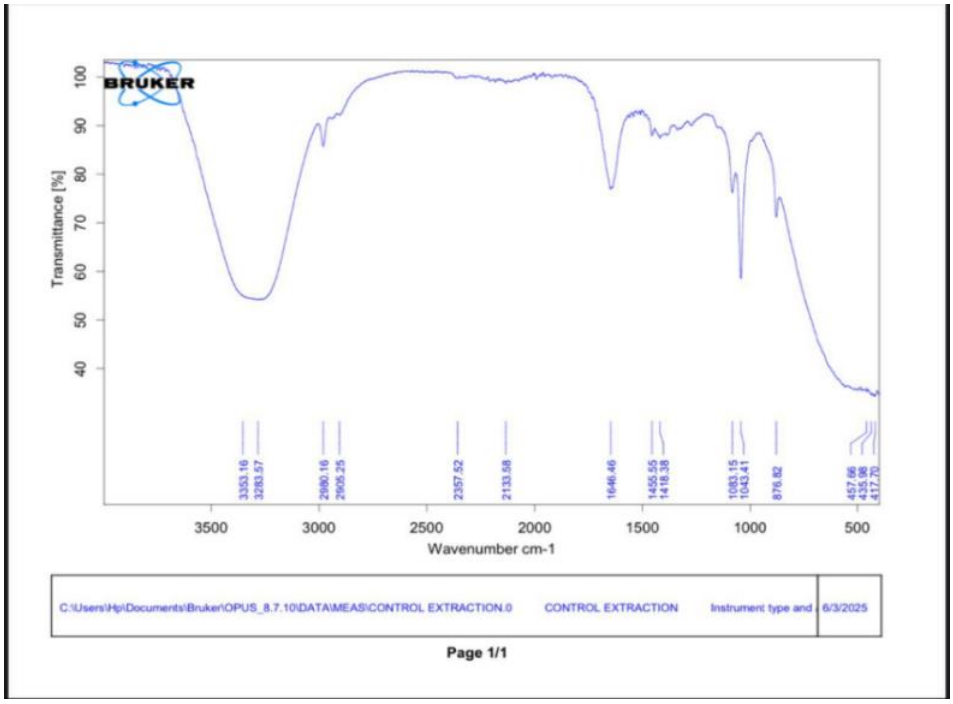


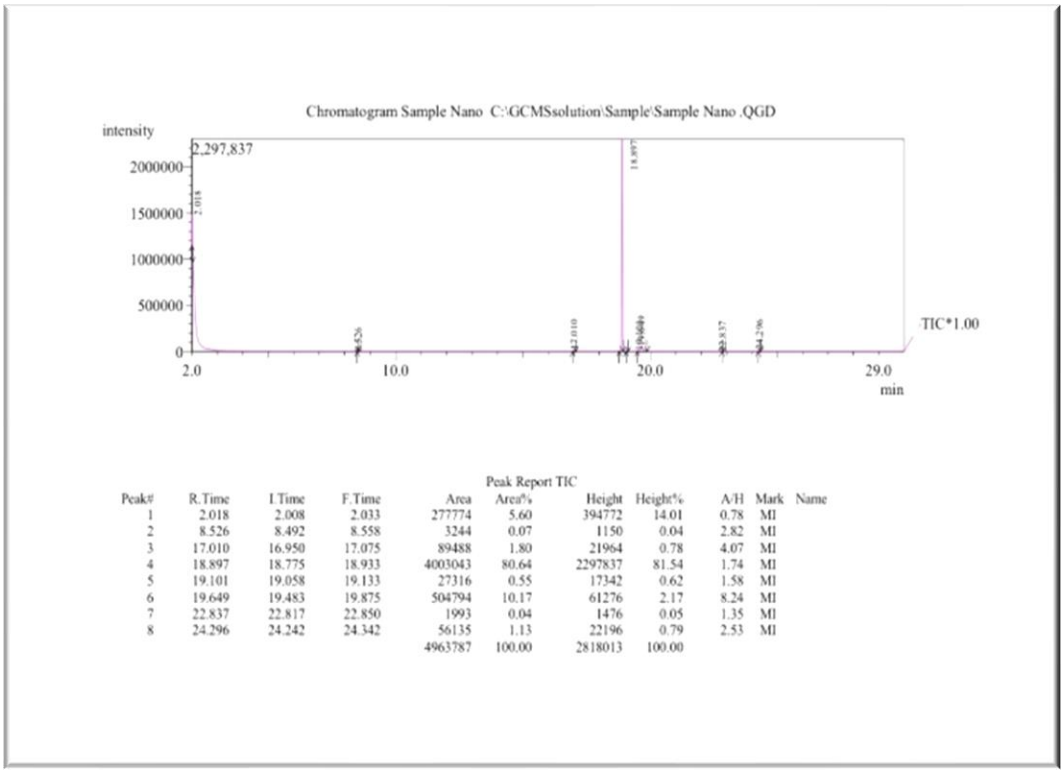
Figure 3. Wax Formed from Wax Cells Only (Control)

Analysis of the FT-IR Results

The presence of the nanocompound (silver) alone leads to an increase in absorption without any shift, which indicates that the carbonyl bond absorption has increased due to a stronger dipole moment. This means that the FT-IR absorbance increased in the presence of the nanocompound because of its dipole magnetic properties and smoothness. Furthermore, when silver is incorporated into the nanocompound, the dipole moment increases even more due to bonding, resulting in higher absorption and lower transmittance.

Analysis of Sample Results Using Gas Chromatography–Mass Spectrometry (GC-MS)

The GC-MS technique was employed to identify the active chemical constituents present in the neem seed nano-extract sample. The results revealed several peaks at different retention times (RTs), each representing a distinct compound that was separated and identified. Each peak was analyzed based on its area, which reflects the compound's concentration, and its area percentage (Area%), which indicates the proportion of each compound relative to the total components.



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.018	2.008	2.033	277774	5.60	394772	14.01	0.78	MI	
2	8.526	8.492	8.558	3244	0.07	1150	0.04	2.82	MI	
3	17.010	16.950	17.075	89488	1.80	21964	0.78	4.07	MI	
4	18.897	18.775	18.933	4003043	80.64	2297837	81.54	1.74	MI	
5	19.101	19.058	19.133	27316	0.55	17342	0.62	1.58	MI	
6	19.649	19.483	19.875	504794	10.17	61276	2.17	8.24	MI	
7	22.837	22.817	22.850	1993	0.04	1476	0.05	1.35	MI	
8	24.296	24.242	24.342	56135	1.13	22196	0.79	2.53	MI	
				4963787	100.00	2818013	100.00			

Figure 4. GC-MS Analysis Results of the Neem Seed Nano-Extract Sample

Table 2. Major Compounds in the Neem Seed Nano-Extract Sample According to Retention Time and Peak Area Percentage

Peak	R.Time (min)	Area / Area %	Compound Name	Formula	CAS	MolWeight (g/mol)
			Topotecan	C23H23NO9	0-00-0	421
			Dimethylamine	C2H7N	124-40-3	45
1	2.020	-901292 / 5.60%	1-Propanol‹ 2-amino- (DL-Alaninol)	C3H9NO	6168-72-5	75
			2-Formylhistamine	C6H9N3O	0-00-0	139
			N‹N-Dimethylethanesulfonamide	C4H11NO2S	6338-68-7	137
			2-Propanamine (Isopropylamine)	C3H9N	75-31-0	59
			Topotecan	C23H23NO9	0-00-0	421
2	2.792	9905 / -1.19%	1-Methoxy-2-propanamine	C4H11NO	37143-54-7	89
			(R)-(-)-2-Amino-1-propanol	C3H9NO	35320-23-1	75
			(S)-(+)-2-Amino-1-propanol	C3H9NO	2749-11-3	75
			Cyanoacetic acid	C3H3NO2	372-09-8	85
			Isobutylene epoxide	C4H8O	558-30-5	72
3	3.528	14922 / -1.80%	Borane carbonyl	CH3BO	13205-44-2	42
			Methyl isopropenyl ether	C4H8O	116-11-0	72
			Ethylamine	C2H7N	75-04-7	45
			4-Methyl-4-hepten-3-one	C8H14O	22319-31-9	126
			1-Pentyne‹ 3-ethyl-3-methoxy-	C8H14O	53941-20-1	126
			2-Pyrazoline‹ 5-ethyl-1‹4-dimethyl-	C7H14N2	14339-23-2	126
4	8.529	7308 / -0.88%	2-(4-Methyl-1H-1‹2‹3-triazol-1-yl)ethan-1-amine	C5H10N4	1086601-35-5	126
			1H-Pyrazole‹ 4‹5-dihydro-3-methyl-1-propyl-	C7H14N2	26964-49-8	126
			2-Methyl-1-nitropropane	C4H9NO2	625-74-1	103
			Methyl isocyanate	C2H3NO	624-83-9	57
5	12.307	4704 / -0.57%	Azetidine	C3H7N	503-29-7	57
			1‹3-Propanediamine	C3H10N2	109-76-2	74
			Allylamine	C3H7N	107-11-9	57
			Methyl butanoate	C5H10O2	623-42-7	102
			2-Amino-2-oxoethyl acetate	C4H7NO3	0-00-0	117
6	17.047	5258 / -0.63%	2-Methylbutyric acid	C5H10O2	116-53-0	102
			2-Methylhexanoic acid	C7H14O2	4536-23-6	130
			2-Methylpentanoic acid	C6H12O2	97-61-0	116
			Methyl 11-octadecenoate	C19H36O2	52380-33-3	296
			Methyl 13-octadecenoate	C19H36O2	56554-47-3	296
7	18.874	30135 / -3.63%	(Z)-15-Octadecenoic acid methyl ester	C19H36O2	0-00-0	296
			Methyl oleate	C19H36O2	112-62-9	296
			Methyl undecenoate	C12H22O2	111-81-9	198

Table 3. of GC-MS analysis results of the active nano-compounds in the nano-extract of Neem plant

Compound	Chemical Class	Biological Role
Dimethylamine	Simple Amine	Broad-spectrum antimicrobial against E. coli and S. aureus, inhibits bacterial growth
Propanamine (Isopropylamine)	Aliphatic Amine	Used in pesticides and antimicrobial drugs, disrupts bacterial cell membranes
Topotecan	Semi-synthetic Alkaloid	Anticancer agent, inhibits Topoisomerase I and prevents DNA replication
Cyanoacetic acid	Nitrile Carboxylic Acid	Antibacterial, antifungal, antioxidant, used as catalyst for bioactive compounds
Pyrazoline, 5-ethyl-1,4-dimethyl-2	Heterocyclic (Pyrazoline derivative)	Antibacterial, antioxidant, anti-inflammatory, some derivatives show selective cytotoxicity
Allylamine	Unsaturated Aliphatic Amine	Antifungal, inhibits squalene epoxidase, effective against Tinea infections
2-Methylbutyric acid	Short-chain Carboxylic Acid	Antibacterial, reduces intracellular pH, anti-inflammatory effects
Methyl oleate	Fatty Acid Ester	Antioxidant, anti-inflammatory, antimicrobial, drug carrier for better absorption

Compound Analysis:

1. Dimethylamine: Broad-spectrum antimicrobial against E. coli and S. aureus; disrupts or inhibits bacterial growth.
2. Propanamine (Isopro pylamine)-2: Used in the synthesis of pesticides and antimicrobial drugs; disrupts bacterial cell membranes.
3. Topotecan: Anticancer agent that inhibits Topoisomerase I and prevents DNA replication in cancer cells.
4. Cyanoacetic acid: An intermediate in organic chemistry with antibacterial, antifungal, and antioxidant activity; also used as a catalyst in chemical reactions to produce bioactive compounds.
5. Pyrazoline, 5-ethyl-1,4-dimethyl-2: Exhibits antibacterial, antioxidant, and anti-inflammatory activity; some derivatives show selective toxicity against cancer cells.
6. Allylamine: Antifungal agent that inhibits squalene epoxidase in fungi; effective against Tinea capitis, Tinea corporis, and nail infections.
7. Methylbutyric acid: A short-chain carboxylic acid with antibacterial properties; reduces intracellular pH in microbes and has anti-inflammatory effects.

8. Methyl oleate: Antioxidant, anti-inflammatory, and antimicrobial agent; also used as a drug carrier to enhance drug absorption.

The results were showed that the nanoparticles exhibited either a spherical shape individually or in crystalline aggregates, and that the neem nano-extract was highly effective in eliminating the larvae of the greater wax moth, without causing harmful toxic effects on the environment. This study indicates that plant-based or plant-waste–derived green biosynthesis represents an effective and safe strategy for insect pest management.
Residual Effect of the Samples (Neem Seed Nano-Extract, Wax Cells Treated with the Nano-Extract, and Untreated Wax Cells).

Table 4. Residual Effect for Samples

Sample	Silver concentration (ppm)	Notes
Neem seed nano-extract before treatment	136.7	High concentration reflects the high density of nanoparticles
Wax cells treated with the nano-extract	0.3	Very slight residual effect due to the cleaning behavior of worker bees
Wax cells without treatment	0.0	No concentration detected, only in the treated sample

Results Analysis

The results indicate that the silver concentration in the neem seed nano-extract was high (136.7 ppm), reflecting the high density of nanoparticles in the extract. In contrast, wax cells treated with the nano-extract showed a very low concentration (0.3 ppm), indicating only a slight residual presence of nanoparticles deposited or adsorbed on the wax surface after treatment. This residual effect can be attributed to the ability of some nanoparticles to adhere to the wax surface or penetrate its fine pores. However, the quantity was much lower than that in the original extract, suggesting that most of the silver nanoparticles neither transferred nor accumulated in significant amounts in the wax, and thus the likelihood of cumulative or toxic effects is very low .Meanwhile, untreated wax cells showed no detectable silver concentration, confirming that the source of silver in the second case was solely the treatment with the nano-extract.

Conclusion

The study demonstrated that the nano-extract of neem (Azadirachta indica) enriched with silver nanoparticles (AgNPs) possesses strong biological activity without adversely affecting honeybees or the environment. Quantitative and qualitative analyses (FT-IR, GC-MS, Atomic Absorption) revealed that the extract contains active compounds such as Azadirachtin, Nimbin, Polyphenols, Fatty acids, and spherical silver nanoparticles, which confer antibacterial, antifungal, antioxidant, and insecticidal properties. Residual effect assessment showed that the silver concentration in the nano-extract was initially high (136.7 ppm) but significantly decreased to 0.3 ppm in wax frames after treatment by honeybees,

indicating the bees' ability to remove most of the nanoparticles. Untreated wax cells showed no detectable silver, confirming that the only source of silver was the nano-extract treatment. Based on these findings, it can be concluded that using the neem nano-extract with silver nanoparticles provides a safe and effective method for pest control in wax frames, minimizing environmental impact while preserving human and bee safety.

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