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SYNERGISTIC ACARICIDAL ACTIVITY OF *MELIA AZEDARACH* AND *NIGELLA SATIVA* AGAINST *VARROA DESTRUCTOR* IN *APIS MELLIFERA*: AN IN VIVO STUDY

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Abstract

Varroa destructor is a major ectoparasitic mite threatening Apis mellifera colonies worldwide, causing colony weakening and significant economic losses. With increasing resistance to synthetic acaricides and concerns over chemical residues in hive products, there is a growing need for sustainable and eco-friendly control strategies. This study evaluated the acaricidal and repellent potential of methanolic extracts of Melia azedarach leaves and Nigella sativa seeds, individually and in combination, against Varroa destructor under controlled laboratory conditions. Contact toxicity bioassays revealed dose-dependent mite mortality, with M. azedarach showing the highest efficacy (60–100%) but moderate honeybee toxicity, while N. sativa exhibited moderate mite mortality (22.7–49.3%) with minimal bee mortality. Notably, combined extracts demonstrated synergistic effects, achieving greater-than-predicted mite mortality (53.3%) at lower concentrations, suggesting enhanced efficacy and safety. Repellence assays indicated strong avoidance behavior, particularly for M. azedarach, highlighting the potential dual mode of action. These findings indicate that botanical combinations can serve as effective, environmentally safe alternatives to conventional acaricides, contributing to integrated Varroa management and sustainable apiculture.

INTRODUCTION

The western honeybee, *Apis mellifera*, is a highly organized social insect belonging to the family Apidae under the order Hymenoptera. Honeybees exist in complex and cooperative colonies and represent one of the most ecologically and economically important insect groups due to their pollination services and valuable hive products. With more than 20,000 described bee species distributed worldwide, honeybees have long attracted human interest because of their essential contributions to agriculture, biodiversity, and

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food security (Shaher and Awwad, 2023). In particular, *A. mellifera* plays a fundamental role in sustaining ecosystems and agricultural productivity through pollination while also providing commercially important products such as honey, wax, propolis, and pollen (Papa *et al.*, 2022). Insect-mediated pollination is estimated to increase global crop production by 15–30% by improving both yield and quality, especially in fruits, vegetables, oilseed crops, nuts, and spices, while also supporting the reproduction of wild plant species and maintaining biodiversity (Gallai *et al.*, 2009; Papa *et al.*, 2022).

Despite their importance, honeybees face numerous biological threats, including pathogens and parasites such as fungi, bacteria, viruses, and various insect and non-insect pests (Shaher and Awwad, 2023). Among these, the ectoparasitic mite *Varroa destructor* is considered the most destructive pest affecting honeybee colonies globally. Formerly classified as *Varroa jacobsoni* until taxonomic differentiation in 2000, *V. destructor* is now recognized as the primary driver of global honeybee colony losses (Anderson and Trueman, 2000; Warner *et al.*, 2024). The mite parasitizes honeybees at multiple developmental stages including larvae, pupae, and adults, spreading through mechanisms such as brood transfer, drifting bees, robbing behavior, and colony migration (Al-Hasnawi, 2019). Rather than feeding solely on hemolymph as previously believed, *V. destructor* primarily targets the fat body tissues of developing and adult bees, thereby weakening physiological functions and facilitating the transmission of pathogenic viruses (Ramsey *et al.*, 2019).

The mite is also an efficient vector of several viral pathogens, particularly Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV), which severely affect brood development, adult bee longevity, and queen health, ultimately threatening colony survival (Amiri *et al.*, 2020). Infestation by *V. destructor* suppresses the immune response of honeybees, disrupts metabolic activity, shortens lifespan, and reduces foraging efficiency and pollination capacity (Traynor *et al.*, 2020; Muijres *et al.*, 2020). Consequently, Varroa infestation contributes indirectly to colony weakening and population decline (Mehmood, 2021). The parasite also promotes secondary infections by creating wounds that remain open for extended periods, facilitating pathogen entry and further weakening colony health (Morfin *et al.*, 2023).

The biology and reproductive strategy of *V. destructor* further contribute to its destructive capacity. Female mites reproduce within sealed brood cells, preferentially targeting drone brood due to its longer developmental period, which provides favorable conditions for mite reproduction (Boecking and Genersch, 2008). After entering brood cells prior to capping, the female mite lays eggs that develop through several nymphal stages, feeding on developing bee pupae. This parasitism often results in deformed wings, reduced body weight, shortened lifespan, and impaired flight ability in emerging adult bees (Mehmood, 2021). Transmission between colonies occurs through drifting workers, robbing behavior, swarming, and movement of drones, making effective management particularly challenging (Peck and Seeley, 2019; Mortensen *et al.*, 2018).

Various control strategies have been developed to manage *V. destructor*, including mechanical, biological, and chemical approaches. Mechanical methods such as screened bottom boards, drone brood removal, and thermal treatments have shown some effectiveness in reducing mite populations, although they are generally insufficient when used alone (Rosenkranz *et al.*, 2010). Biological control approaches involving entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have demonstrated promising acaricidal effects, although potential impacts on bee brood remain a concern (Meikle *et al.*, 2012; Sinia and Guzman-Novoa, 2018). Similarly, certain bacterial agents such as *Bacillus thuringiensis* have shown potential in laboratory studies, achieving significant mite mortality (Alquisira-Ramirez *et al.*, 2014).

Chemical control remains the most widely adopted strategy, particularly through the use of synthetic acaricides such as amitraz, coumaphos,

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fluvalinate, and flumethrin due to their high efficacy and ease of application (Rosenkranz *et al*., 2010). However, the repeated and improper use of these compounds has resulted in the development of resistance in mite populations and contamination of hive products including honey and wax (Rosenkranz, 2010). These concerns have encouraged increased interest in safer alternatives such as organic acids and plant-derived compounds. Botanical pesticides are increasingly recognized as environmentally sustainable pest management tools due to their biodegradability, reduced toxicity, and diverse bioactive properties including toxicity, repellency, antifeedant activity, and growth regulation (Aivazi and Vijayan, 2009; Pathak *et al*., 2022).

Plant-based acaricides have attracted significant attention as sustainable alternatives to conventional pesticides. Members of the family Meliaceae are particularly known for their rich composition of bioactive compounds with insecticidal and growth-regulating properties (Nakatani *et al*., 2004). *Melia azedarach*, commonly known as chinaberry or Persian lilac, is widely distributed in tropical and subtropical regions and has demonstrated pesticidal activity against several arthropod pests (Carpinella *et al*., 2003; Nathan and Saehoon, 2005). Extracts from various parts of this plant have shown larvicidal and acaricidal properties, including activity against mosquito larvae and ticks (Wondscheer *et al*., 2004; Borges *et al*., 2003).

Similarly, *Nigella sativa*, commonly known as black seed or black cumin, is a medicinal plant widely distributed across the Mediterranean region and parts of Asia including Pakistan, India, and Iran (Gali-Muhtasib *et al*., 2006). Extracts of *N. sativa* have demonstrated acaricidal and antiparasitic properties in laboratory studies, including mortality effects against Varroa mites and ticks (Aboelhadid *et al*., 2016; Shah *et al*., 2025). Bioactive compounds such as thymoquinone present in *N. sativa* have also shown inhibitory activity against various protozoan parasites, further highlighting its pharmacological and pesticidal potential (El-Sayed *et al*., 2019).

Considering the growing limitations of synthetic acaricides and the need for sustainable pest management strategies, plant extracts offer promising alternatives for Varroa control. The present study therefore aims to evaluate the acaricidal potential of *Melia azedarach* and *Nigella sativa* extracts as safer and environmentally friendly alternatives for managing *Varroa destructor* infestations in *Apis mellifera*. Furthermore, this research investigates the combined effects of these botanical extracts to determine possible synergistic interactions and to evaluate their safety profile for Honeybee s. The exploration of such plant-based acaricides may contribute to the development of effective, economically viable, and ecologically sustainable strategies for Varroa management while reducing dependence on synthetic chemicals.

The specific objectives of this study include evaluating the synergistic acaricidal effects of methanolic extracts of *Melia azedarach* and *Nigella sativa* against *Varroa destructor*, and assessing the attraction or repellent responses of adult mites to selected botanical extracts using a two-choice static air bioassay.

MATERIALS AND METHODS

2.1. Study design

The present study was conducted under controlled laboratory conditions to evaluate the acaricidal and repellent potential of *Melia azedarach* and *Nigella sativa* extracts against *Varroa destructor*, with particular emphasis on their toxicity against mites and safety toward *Apis mellifera*. The experimental work was carried out at the Department of Zoology, Kohat University of Science and Technology (KUST), Kohat, Pakistan, during the period from October to December 2025.

The experimental design consisted of two major components. The first component involved a contact toxicity bioassay designed to evaluate the acaricidal efficacy of individual plant extracts and their combined formulations at different concentration levels, while simultaneously assessing their safety profile for Honeybee s. The second component consisted of a repellence bioassay aimed at determining the behavioral responses of *Varroa destructor* to plant extracts

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using a two-choice static air system. Both experiments were performed under standardized laboratory conditions to ensure reproducibility and minimize environmental variability.

2.2. Ethical approval

Prior to the initiation of the experimental work, ethical approval was obtained from the Research Ethical Committee of Kohat University of Science and Technology (KUST), Kohat, Pakistan. All experimental procedures were conducted in accordance with institutional biosafety and ethical guidelines for research involving invertebrates. Standard apicultural handling procedures were followed throughout the study to minimize stress, injury, and unnecessary mortality of Honeybee s during collection, handling, and experimentation.

2.3. Collection and authentication of plant materials

2.3.1. Collection of *Melia azedarach* leaves

Fresh and healthy leaves of *Melia azedarach* were collected from their natural habitat in the Kohat region of Khyber Pakhtunkhwa, Pakistan. Leaves were carefully selected from mature and disease-free trees to ensure sample quality. Plant material showing symptoms of pest infestation, pathogen infection, or physical damage was excluded from the collection to maintain consistency and reliability of the experimental material.

2.3.2. Procurement of *Nigella sativa* seeds

Certified seeds of *Nigella sativa* (family Ranunculaceae) were procured from a reputable local herbal market in Kohat city, Khyber Pakhtunkhwa, Pakistan. Seeds were selected based on uniformity in size and characteristic black coloration. Care was taken to ensure that the seeds were free from foreign particles, physical damage, and visible fungal contamination to maintain the integrity of the experimental samples.

2.3.3. Authentication of plant materials

Both plant species were taxonomically authenticated by a qualified botanist at the Department of Botany, Kohat University of Science and Technology. Authentication was performed based on standard morphological characteristics to confirm species identity prior to extraction and experimental use.

2.4. Preparation of plant extracts

2.4.1. Sample processing

The collected leaves of *M. azedarach* were thoroughly washed under running tap water to remove adhering dust and contaminants, followed by rinsing with distilled water to eliminate residual impurities. The leaves were then shade-dried at room temperature (25 ± 2°C) for approximately 8–10 days until a constant dry weight was achieved. Shade drying was preferred to prevent degradation of heat-sensitive bioactive compounds.

The seeds of *N. sativa* were manually cleaned to remove dust and extraneous materials and were not washed in order to preserve potentially important surface bioactive constituents. After drying, both plant materials were separately ground into fine powder using an electric grinder. The powdered material was passed through a fine mesh sieve to obtain uniform particle size. The processed samples were stored in airtight amber glass containers at 4°C until further extraction to prevent degradation and photochemical reactions.

2.4.2. Methanolic extraction procedure

Methanolic extracts were prepared following the procedure described by Shah *et al.* (2025) with minor modifications. Briefly, 100 g of powdered plant material was mixed with 400 mL of analytical grade methanol at a ratio of 1:4 (w/v) in sterile glass flasks. The mixtures were tightly sealed and allowed to macerate at room temperature (25 ± 2°C) for five days. Intermittent shaking was performed twice daily to enhance the extraction efficiency of bioactive compounds.

After the extraction period, the mixtures were filtered through Whatman No. 1 filter paper (11 µm pore size) to remove plant debris. The filtrates were then concentrated using a rotary evaporator at 40°C under reduced pressure to remove the solvent and obtain semi-solid crude extracts. The concentrated extracts were transferred into pre-weighed amber glass vials and stored at 4°C until further experimental use.

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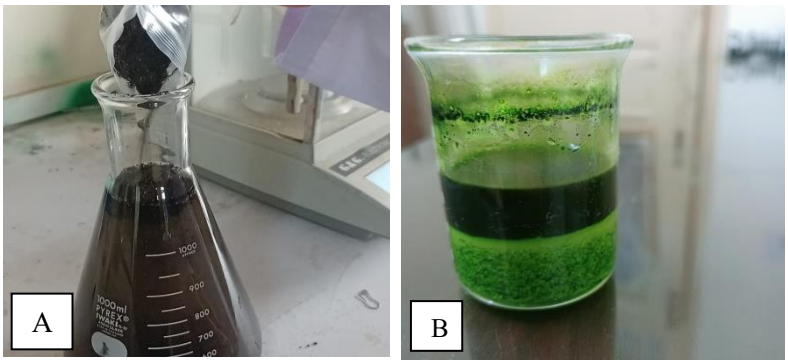

Figure 2.1: Preparation of methanolic extracts from medicinal plants: (A) *Nigella sativa* and (B) *Melia azedarach*, showcasing the initial steps in phytochemical extraction for bioactive compound analysis

2.4.3. Preparation of stock solutions and working concentrations

Stock solutions of plant extracts were prepared at a concentration of 200 g/L (20% w/v) by dissolving the required quantity of crude extract in distilled water. From these stock solutions, working concentrations of 50 g/L (5%), 100 g/L (10%), and 200 g/L (20%) were prepared through serial dilution using distilled water. All solutions were prepared freshly on the day of application to maintain stability of the active constituents.

For combination treatments, equal volumes of *M. azedarach* and *N. sativa* extracts were mixed to obtain final combined concentrations of 25+25 g/L, 50+50 g/L, and 100+100 g/L respectively.

2.5. Collection of mite-infested Honeybees

2.5.1. Source of bees

Worker bees of *Apis mellifera* naturally infested with *Varroa destructor* were collected from local apiaries in the Kohat region. Local beekeepers were consulted to identify colonies showing visible symptoms of Varroa infestation. Colonies exhibiting moderate to high infestation levels were selected as source colonies to ensure adequate mite availability for experimentation.

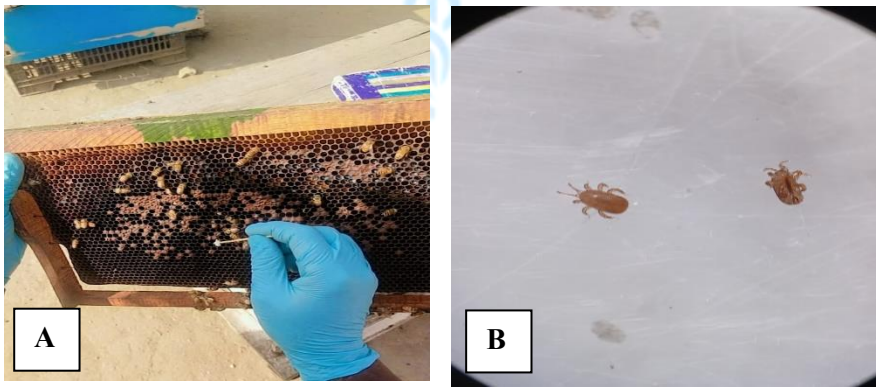

Figure 2.2 A) Collection of mites from an infected honeybee colony; B) Microscopic identification of *Varroa destructor* isolated from the infected colony

2.5.2. Collection procedure

Adult worker bees were carefully collected from brood frames and transferred into ventilated plastic containers measuring approximately 20 × 15 × 10 cm. The containers were fitted with mesh sides to allow ventilation during transport. Twenty-five bees were placed in each container and immediately transported to the laboratory for experimentation.

To maintain natural infestation levels and avoid experimental bias, no artificial infestation with mites was performed. Naturally infested bees were

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directly used in the bioassays.

2.6. Experimental setup for contact toxicity bioassay

2.6.1. Ventilated container system

The contact toxicity bioassay was performed using a modified ventilated container system based on the methodology described by Bahreini *et al.* (2021). Transparent plastic containers measuring approximately 15 cm in height and 10 cm in diameter were used. Each container was fitted with a screw cap and modified by installing a double-layer mesh (2 mm aperture) horizontally at a distance of 5 cm from the top, thereby dividing the container into two compartments.

Figure 2.3 A) Ventilated container system for contact toxicity bioassay

The upper compartment was used for holding bees and applying treatments, while the lower compartment served to collect fallen mites. Small ventilation holes were made in the container walls to allow sufficient air exchange while preventing mite escape. A small quantity of sugar particles was placed inside the containers as a food source for bees during the experimental period.

2.6.2. Acclimatization of bees

Prior to treatment application, twenty-five worker bees were introduced into the upper compartment of each container and allowed to acclimatize for one hour under laboratory conditions maintained at 25 ± 2°C and 60 ± 5% relative humidity. This acclimatization period allowed bees to recover from handling stress and ensured more consistent experimental responses.

2.6.3. Treatment application

Treatment solutions were applied by spraying according to the procedure described by Shaher *et al.* (2023). A measured quantity of each treatment solution was uniformly sprayed onto the bees in the upper chamber to ensure adequate exposure. Care was taken to apply treatments consistently across all experimental groups.

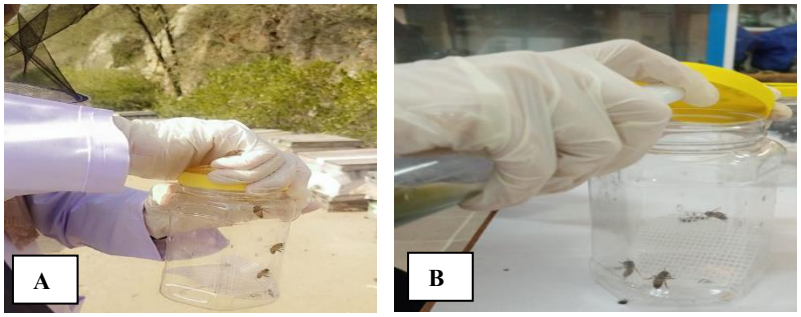

Figure 2.4: A) Collection of mite-infected bees; B) Application of plant extract using a sprayer for treatment."

2.6.4. Experimental groups

The experiment consisted of five treatment groups, each comprising 25 mite-infested bees, with three replicates maintained for each

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treatment to ensure statistical reliability. The first group served as a negative control and was treated with distilled water. The second group served as a positive control and was treated with amitraz at a concentration of 0.5 g/L to provide a reference for standard acaricidal efficacy.

The third and fourth groups were treated with methanolic extracts of *M. azedarach* leaves and *N. sativa* seeds, respectively, while the fifth group received a combined formulation of both extracts. Each plant extract treatment was tested at three concentrations (5%, 10%, and 20%) to determine the most effective lethal concentration. All experiments were conducted in triplicate to improve data reliability and reproducibility.

2.6.5. Post-treatment observations

Following treatment application, the containers were maintained under controlled laboratory conditions for 24 hours. After this exposure period, mite mortality and bee mortality were recorded. Mite mortality was determined by counting the number of mites that had fallen into the lower compartment. Mites were considered dead if they showed no movement when gently stimulated with a fine brush.

Bee mortality was determined by counting dead individuals in the upper compartment. Bees were considered dead if they showed no movement of antennae or appendages upon gentle physical stimulation. These observations were used to evaluate both acaricidal efficacy and relative safety of the treatments.

2.7. Repellence bioassay

2.7.1. Two-choice static air apparatus

The repellent activity of plant extracts against *Varroa destructor* was evaluated using a two-choice static air bioassay adapted from standard behavioral testing methods. The apparatus consisted of a glass Petri dish of 10 cm diameter lined with Whatman No. 1 filter paper discs measuring 5 cm in diameter. Each filter paper disc was cut into two equal halves. One half was treated with 1 mL of plant extract solution at 100 g/L concentration, while the other half was treated with 1 mL of distilled water to serve as the control. The treated and control filter paper halves were placed on opposite sides of the Petri dish to create a choice arena. Ten adult female *V. destructor* mites were carefully collected from infested bees using a fine camel hair brush. Individual mites were placed at the center line of the Petri dish and allowed to move freely for 30 minutes under dim light conditions to minimize phototactic effects.

After the observation period, the position of each mite was recorded to determine attraction or repellence responses toward the tested extracts. The distribution of mites between treated and untreated areas was used as an indicator of repellent activity.

RESULTS

3.1. Contact toxicity of individual extracts against *Varroa destructor*

The acaricidal activity of *Melia azedarach* and *Nigella sativa* extracts was evaluated individually at three concentrations (50, 100, and 200 g/L) against *Varroa destructor* infesting *Apis mellifera*. Mortality observations were recorded 24 hours after treatment application. The results are presented as mean values obtained from three independent replicates, each consisting of 25 bees.

3.1.1. Contact toxicity of *Melia azedarach* extract

Table 3.1: Mite mortality following treatment with *Melia azedarach* extract

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf. Interval (Lower)	Upper
50	15	16	14	15±0.89	60%	0.0015	12.516	17.484
100	18	17	19	18±0.89	72%	0.001	15.516	20.484
200	25	25	25	25±0	100%	NA	NA	NA

The results demonstrated that *Melia azedarach* extract exhibited strong acaricidal activity in a clear

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dose-dependent manner. At the lowest tested concentration of 50 g/L, the mean mite mortality was 60.0%, corresponding to 15.00 ± 0.89 mites. Increasing the concentration to 100 g/L resulted in an increase in mortality to 72.0%, corresponding to 18.00 ± 0.89 mites. Complete mortality (100%) was observed at the highest concentration of 200 g/L, where all mites were killed in each replicate.

3.1.2. Contact toxicity of *Nigella sativa* extract

Table 3.2: Mite mortality following treatment with *Nigella sativa* extract

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf. Interval (Lower)	Upper
50	5	6	6	5.67±0.52	23%	0.0034	4.2324	7.1009
100	10	11	10	10.33±0.52	41%	0.001	8.8991	11.768
200	12	13	12	12.33±0.52	49%	0.0007	10.899	13.768

The results indicated that *Nigella sativa* extract exhibited moderate acaricidal activity against *V. destructor*. At 50 g/L, the mortality rate was 22.7% (5.67 ± 0.52 mites). This increased to 41.3% (10.33 ± 0.52 mites) at 100 g/L. The highest mortality of 49.3% (12.33 ± 0.52 mites) was observed at 200 g/L, indicating a concentration-dependent increase in efficacy, although the overall activity remained lower than that observed for M. *azedarach*.

3.2. Individual extract treatments and bee safety

To evaluate the safety of the botanical extracts for non-target organisms, mortality of adult Honeybees was recorded alongside mite mortality.

3.2.1. Bee mortality following *Melia azedarach* treatment

Table 3.3: Honeybee mortality following treatment with *Melia azedarach* extract

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf Interval (Lower)	Upper
50	5	6	5	5.33±0.52	21.3%	0.0039	3.8991	6.7676
100	12	11	13	12±0.89	48%	0.0023	9.5159	14.484
200	15	14	16	15±0.89	60%	0.0015	12.516	17.484

Melia azedarach extract caused notable mortality in Honeybee s in a dose-dependent manner. At 50 g/L, bee mortality was 21.3% (5.33 ± 0.52 bees). Mortality increased to 48.0% (12.00 ± 0.89 bees) at 100 g/L and further increased to 60.0% (15.00 ± 0.89 bees) at 200 g/L. The relatively high bee mortality observed at concentrations that were effective in controlling mites suggests that M. *azedarach* extract alone may have limited safety as a treatment option.

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3.2.2. Bee mortality following *Nigella sativa* treatment

Table 3.4: Honeybee mortality following treatment with *Nigella sativa* extract

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf Interval (Lower)	Upper
50	0	0	0	0±0	0%	NA	NA	NA
100	0	0	1	0.33±0.52	1.3%	0.4226	-1.1009	1.7676
200	2	1	1	1.33±0.52	5.3%	0.0572	-0.1009	2.7676

Nigella sativa extract demonstrated excellent safety toward Honeybee s. No bee mortality was observed at 50 g/L. At 100 g/L, only minimal mortality (1.3%) was recorded, while at 200 g/L mortality remained low at 5.3%. The differences in bee mortality between *M. azedarach* and *N. sativa* treatments were highly significant ($p < 0.001$), highlighting the superior safety profile of *N. sativa*.

3.3. Contact toxicity of combined *M. azedarach* and *N. sativa* extracts

To investigate potential synergistic effects, combined extracts of *M. azedarach* and *N. sativa* were tested at three concentrations (25+25, 50+50, and 100+100 g/L) and compared with the effects of individual treatments.

3.3.1. Mite mortality following combination treatment

Table 3.5: Mite mortality following treatment with MA + NS combination

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf Interval (Lower)	Upper
50	13	14	13	13.33±0.52	53%	0.0006	11.899	14.768
100	20	19	21	20±0.89	80%	0.0008	17.516	22.484
200	23	24	22	23±0.89	92%	0.0006	20.516	25.484

The combined extracts demonstrated enhanced acaricidal efficacy at all tested concentrations. At the lowest concentration (25+25 g/L), mite mortality reached 53.3% (13.33 ± 0.52 mites). At 50+50 g/L, mortality increased to 80.0% (20.00 ± 0.89 mites), while the highest concentration (100+100 g/L) resulted in 92.0% mortality (23.00 ± 0.89 mites), indicating improved performance compared to individual *N. sativa* treatment.

3.3.2. Bee mortality following combination treatment

Table 3.6: Honeybee mortality following treatment with MA + NS combination

Concentration (g/L)	Replicate 1	Replicate 2	Replicate 3	Mean (SD)	Mean (%)	P value	95% Conf. Interval (Lower)	Upper
50	0	0	0	0±0	0%	NA	NA	NA
100	5	4	6	5±0.89	20%	0.0131	2.5159	7.4841
200	7	8	7	7.33±0.52	29.3%	0.0021	5.8991	8.7676

The safety profile of the combined treatment was improved compared to *M. azedarach* alone. At 25+25 g/L, mite mortality of 53.3% was achieved with zero bee mortality. At 50+50 g/L, mite

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mortality reached 80.0% with 20.0% bee mortality. At 100+100 g/L, mite mortality reached 92.0% with 29.3% bee mortality.

The 25+25 g/L combination appeared to provide an optimal balance between efficacy and safety, achieving substantial mite control with no observed bee mortality. This represents an improvement over individual treatments, where *M. azedarach* showed safety concerns and *N. sativa* showed lower efficacy.

3.4. Bee and mite mortality in control groups

In the control groups, distilled water produced no mortality in either bees or mites, confirming that experimental handling and environmental conditions did not influence mortality outcomes. In contrast, amitraz applied at its standard concentration of 0.5 g/L resulted in 80% mite mortality and 10% bee mortality, confirming its strong acaricidal efficacy and validating its use as a positive control.

3.5. Repellence assay results

The repellence activity of individual and combined plant extracts was evaluated using a two-choice static air bioassay. Mite distribution between treated and untreated surfaces was recorded after 30 minutes.

3.5.1. Repellence of *Melia azedarach* extract

Table 3.7: Repellence activity of *Melia azedarach* extract

Replicate	Control Side (Mites)	Treated Side (Mites)	Control (%)	Treated (%)	P value
1	9	1	90%	10%	0.1223
2	8	2	80%	20%	
3	9	1	90%	10%	
Mean	8.67	1.33	86.7%	13.3%	

Melia azedarach extract demonstrated strong repellent activity against *V. destructor*, with 86.7% of mites preferring the untreated surface and only 13.3% remaining on treated surfaces. This indicates strong avoidance behavior toward the extract.

3.5.2. Repellence of *Nigella sativa* extract

Table 3.8: Repellence activity of *Nigella sativa* extract

Replicate	Control Side (Mites)	Treated Side (Mites)	Control (%)	Treated (%)	P value
1	6	4	60%	40%	0.0396
2	5	5	50%	50%	
3	7	3	70%	30%	
Mean	6	4	60%	40%	

Nigella sativa extract showed moderate repellent activity, with 60% of mites preferring the control surface compared to 40% on treated surfaces. This difference was statistically significant ($p < 0.05$).

3.5.3. Repellence of *M. azedarach* + *N. sativa* combination

Table 3.9: Repellence activity of *M. azedarach* + *N. sativa* combination

Replicate	Control Side (Mites)	Treated Side (Mites)	Control (%)	Treated (%)	P value
1	8	2	80%	20%	0.2921
2	7	3	70%	30%	
3	8	2	80%	20%	
Mean	7.67	2.33	76.7%	23.3%	

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The combined extracts demonstrated considerable repellent activity, with 76.7% of mites preferring the control surface. This repellence level was intermediate between the strong repellence of *M. azedarach* and the moderate repellence of *Nigella sativa*, suggesting that *M. azedarach* contributes substantially to the behavioral effects observed in the combination.

3.6. Therapeutic index analysis

The therapeutic index (TI) was calculated to assess the balance between acaricidal efficacy and bee safety. Higher TI values indicate more favorable safety-efficacy relationships.

Table 3.10: Therapeutic index values for all treatments

Treatment	Concentration (g/L)	Mite Mortality (%)	Bee Mortality (%)	TI	P value
MA	50	60	21.3	2.8	
MA	100	72	48	1.5	
MA	200	100	60	1.7	
NS	50	22.7	0	∞	<0.01
NS	100	41	1.3	31.6	
NS	200	49.3	5.3	9.3	
NS+MA	25+25	53	0	∞	
NS+MA	50+50	80	20	4.0	
NS+MA	100+100	92	29.3	3.1	

Therapeutic index analysis identified the 25+25 g/L combination as the most balanced treatment, achieving substantial mite mortality with zero bee mortality. The 50+50 g/L treatment provided higher efficacy with acceptable safety, while the 100+100 g/L treatment provided near-complete control but with increased bee mortality.

CONCLUSION

The present study demonstrated that the botanical extracts of *Melia azedarach* and *Nigella sativa* possess significant acaricidal potential against *Varroa destructor*, although their efficacy and safety profiles differed considerably. *Melia azedarach* exhibited strong dose-dependent acaricidal activity, achieving up to 100% mite mortality at the highest concentration. However, this high efficacy was accompanied by considerable honeybee mortality, indicating a narrow safety margin. These findings are consistent with previous reports describing the insecticidal and acaricidal properties of *M. azedarach*, which are mainly attributed to the presence of limonoids and other bioactive secondary metabolites. Despite its effectiveness against mites, the relatively high bee mortality observed suggests that the standalone use of *M. azedarach* extract may pose risks to colony health if not carefully optimized.

In contrast, *Nigella sativa* extract showed moderate acaricidal activity but demonstrated excellent safety toward honeybees, with very low mortality even at higher concentrations. This indicates that *N. sativa* may serve as a safer botanical alternative, although its comparatively lower mite mortality suggests limited effectiveness when used alone. The moderate repellence observed in this study further indicates that its role may be more supportive rather than primary in mite management strategies. The safety profile of *N. sativa* could be linked to its well-known antioxidant and bioactive compounds such as thymoquinone, which may contribute to selective toxicity toward mites while sparing bees.

Importantly, the combined application of *M. azedarach* and *N. sativa* extracts produced improved outcomes, demonstrating a balance between efficacy and safety. The combination treatments showed enhanced mite mortality compared to *N. sativa* alone, while simultaneously reducing bee mortality compared to *M. azedarach* alone. The 25+25 g/L combination was particularly notable, as it achieved substantial mite control with zero bee mortality, resulting in the

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most favorable therapeutic index. This suggests a possible synergistic interaction between the two plant extracts, where *M. azedarach* provides strong acaricidal action while *N. sativa* may help moderate toxicity toward bees. These findings highlight the potential of botanical combinations as eco-friendly alternatives to synthetic acaricides such as amitraz, which, although effective, may lead to resistance development and residue concerns. Overall, the study supports the potential integration of these botanical extracts, particularly in combination, into sustainable *Varroa* management programs.

RECOMMENDATIONS

Based on the findings of the present study, further research should focus on validating these results under field conditions. Since this work was conducted under controlled laboratory conditions using small groups of bees, future experiments should be performed in full-sized honeybee colonies to better reflect natural hive conditions, including variations in temperature, humidity, and colony dynamics. Such field evaluations would help determine the practical applicability of *Melia azedarach* and *Nigella sativa* extracts, particularly their combined use, for integrated *Varroa destructor* management. Additionally, long-term studies are recommended to assess colony-level impacts such as brood development, queen performance, honey production, overwintering success, and overall colony strength, which were beyond the scope of the present short-term toxicity study.

Further optimization of the combined treatment is also recommended. The present study evaluated only a fixed 1:1 ratio of *M. azedarach* and *N. sativa* extracts, but different ratios may produce stronger synergistic effects with improved safety profiles. Therefore, future studies should investigate multiple extract ratios using advanced approaches such as isobolographic analysis to identify the most effective and safest combination. Moreover, biochemical and molecular investigations are needed to better understand the mechanisms responsible for the observed acaricidal and repellent effects in *V. destructor*. Understanding the mode of action would not only strengthen the scientific basis of these botanical treatments but also support their development as reliable alternatives to conventional synthetic acaricides.

Finally, applied research should focus on formulation development and safety validation to support practical adoption. Development of improved delivery systems such as microencapsulation, emulsifiable concentrate (EC) formulations, or the use of suitable adjuvants may enhance stability, prolong activity, and improve field efficacy of the botanical combinations. At the same time, detailed residue analysis in honey, beeswax, and pollen using sensitive analytical techniques such as LC-MS/MS and GC-MS is necessary to confirm product safety and facilitate regulatory approval. Future research should also examine possible sublethal effects on Honeybee behavior, including foraging efficiency, learning ability, communication behavior, and navigation, to ensure that these treatments remain safe not only in terms of mortality but also in maintaining normal colony functioning.

DISCUSSION

The plastic container bioassay employed in this study proved to be a simple, economical, and rapid screening method for evaluating the acaricidal potential of botanical extracts against *Varroa destructor*. The results clearly demonstrated dose-dependent acaricidal activity for all treatments, with *Melia azedarach* showing the highest efficacy (60–100% mortality) and *Nigella sativa* exhibiting comparatively moderate activity. The strong acaricidal activity of *M. azedarach* observed in this study is consistent with previous reports on members of the Meliaceae family, which are known to possess potent bioactive compounds with insecticidal and acaricidal properties (Al-Rubae, 2009). Our findings further extend this evidence by confirming its effectiveness specifically against *V. destructor*, one of the most economically important ectoparasites of Honeybee s. Similar acaricidal effects have been reported against related mite species such as *Tetranychus urticae*, where significant toxicity and repellence effects of *M. azedarach* essential oil were demonstrated (Hashemi *et al.*, 2022). The strong repellence observed in the present study (86.7% avoidance)

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further supports the hypothesis that *M. azedarach* may act through multiple mechanisms including contact toxicity and behavioral modification.

Despite its strong efficacy, the significant Honeybee mortality associated with *M. azedarach* (21.3–60%) represents an important limitation. These findings agree with Ezzahra & Hamid (2025), who reported dose-dependent toxicity of *M. azedarach* extracts toward *Apis mellifera*, highlighting the potential risks of its direct application in beekeeping systems. The narrow safety margin observed in the present study, particularly the high bee mortality (48%) at concentrations required for effective mite control (72% mortality at 100 g/L), indicates that *M. azedarach* alone may not be suitable for direct application without modification. In contrast, *N. sativa* demonstrated moderate acaricidal activity (22.7–49.3%) but excellent safety, with minimal bee mortality (0–5.3%), which is consistent with previous studies reporting its relatively lower toxicity to non-target organisms (Abed and Salim, 2020; Shah *et al*., 2025). This contrast between efficacy and safety highlights the classical challenge in botanical pesticide development—balancing effective parasite control with host safety.

The most significant outcome of this study was the demonstration of synergistic interaction between *M. azedarach* and *N. sativa* extracts. The combined treatment achieved higher-than-expected mortality, particularly at the 25+25 g/L concentration, where the observed mortality (53.3%) exceeded the predicted additive response, indicating true synergistic interaction. Similar positive interactions among botanical combinations have been reported by Aglagane *et al*. (2022), who demonstrated improved safety and efficacy of essential oil mixtures against *V. destructor*. Such synergistic combinations may also play an important role in resistance management, as multi-component botanical formulations can target multiple physiological pathways, thereby reducing the likelihood of resistance development compared to single-target synthetic acaricides such as amitraz and fluvalinate. Furthermore, the repellence properties observed in this study align with previous findings (Damiani *et al*., 2010) and suggest that combining acaricidal and repellent effects could provide a dual mode of action for improved *Varroa* control. Taken together, these findings support the potential of botanical combinations as sustainable and environmentally safer alternatives for integrated *Varroa* management, although further validation under field conditions remains necessary.

Author's Certificate

The authors hereby declare that they have no financial, personal, or professional conflicts of interest that could inappropriately influence the content of this research paper. All aspects of the study, including data collection, analysis, interpretation, and reporting, have been conducted objectively and with full academic integrity. The authors affirm that the research findings presented are original and unbiased, and no external party has influenced the results or conclusions of this work.

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