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Research Article

Assessing the Impact of Essential Oils and *Metarhizium anisopliae* on Controlling *Spodoptera litura* (Lepidoptera: Noctuidae)

Rafia Umar*, Muhammad Irfan Ullah, Muhammad Arshad

Department of Entomology, University of Sargodha, 40100, Sargodha, Pakistan

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RU designed and conducted the experiments, collected data, and prepared the initial draft. MIU supervised the study, provided guidance and critically reviewed the manuscript. MA assisted in experimental work, data analysis, and manuscript writing.

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Biocontrol, Botanical insecticides, Essential Oils, *Mentha piperita*, *Metarhizium anisopliae*, *Spodoptera litura*, Toxicity

Abstract | *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is a major polyphagous insect pest responsible for substantial yield losses in numerous agricultural crops. Extensive use of chemical pesticides has led to resistance development in insect populations and raised environmental and human health concerns. This study evaluated the insecticidal efficacy of three essential oils (EOs)-*Citrus sinensis*, *Cymbopogon citratus*, and *Mentha piperita*) and the entomopathogenic fungus (EPF), *Metarhizium anisopliae* as eco-friendly alternatives for managing *S. litura*. Bioassays were conducted on third instar larvae using leaf-dip exposure for essential oils (1.0%, 3.0%, 5.0%) and topical application for EPF concentrations (10^7, 10^8, and 10^9 cfu/mL). Among the tested treatments, *M. piperita* exhibited the highest larval mortality (83.3%) at 5.0% concentration after 120 h. *C. citratus* and *C. sinensis* showed 70.0% and 50.0% mortality, respectively. Meanwhile, *M. anisopliae* achieved 63.3% mortality at the highest concentration (10^9 cfu/ml) after 10 days of application. These findings highlight the potential of *M. piperita* and *M. anisopliae* as sustainable components of integrated pest management (IPM) programs. Their use could reduce dependence on synthetic insecticides and contribute to climate-resilient and environmentally safe crop protection strategies.

Novelty Statement | Comparative evaluation of plant oils and *Metarhizium anisopliae* fungus against *Spodoptera litura* highlights their potential as eco-friendly pest control agents. The research identifies *Mentha piperita* oil as the most effective botanical insecticide. Integration of *M. piperita* and *M. anisopliae* offers a novel, sustainable approach to reduce chemical pesticide dependence in integrated pest management (IPM) programs.

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Introduction

Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae) considered the most devastating pest of agricultural crops around the globe. *S. litura* widely distributed in many countries like Pakistan, Australia, India, America, China, and Africa EFSA et al. (2019) and feeds on more than 250 host plants such as tobacco, eggplant, cabbage, soybean, corn, pepper, and cotton Supriya *et al.* (2018). It voraciously consumes the leaves of plants, resembling animal grazing, causes extensive damage in its later stages, and ultimately leads to crop decay Saraswathi *et al.* (2023). *S. litura* caused up to 75% yield loss in *Capsicum annum*, and 100% in *Capsicum frutescens* Sharma *et al.* (2022). It has ability to survive in unfavorable conditions as it develops resistance against pesticides and has a greater ability to find hosts Song *et al.* (2024). Its young larvae reduce the value of

Corresponding author: Rafia Umar
rrafiaumar@gmail.com

crops and vegetables both qualitatively and quantitatively.

S. litura have developed high resistance to a broad range of insecticides, including cypermethrin with resistance ratios (RRs) 244-376 Babu and Singh (2022), flubendiamide (25.5-fold), chlorantraniliprole (77.0-fold), cyantraniliprole (27.8-fold) (Che *et al*., 2024), which can lead to huge crop losses. Widespread use of chemicals causes environmental, ecological, and health hazards and increases insect resistance and resurgence, including residue problems Ahmad *et al*. (2024). However, continuous use of pesticides causes high-risk diseases such as cancer, extreme respiratory problems, fetal death, and genetic diseases in humans Ahmad *et al*. (2024). Therefore, developing a sustainable and integrated management strategy to tackle the pest problems is essential. In this context, biopesticides can be used as an alternative to combat insecticidal resistance and persistency Singh *et al*. (2024). The biological management approach involves the administration of beneficial entomopathogenic organisms, as well as the use of biopesticides and bio-stimulants Karthi *et al*. (2024). Bio-pesticides like EOs and EPF show greater promise in decreasing pest populations, particularly during their initial phases Singh and Pandey, (2018).

Essential oils possess diverse biological activities and practical applications, with several studies reporting their pesticidal potential against agricultural pests Singh and Pandey (2018). Plant derived EOs have been shown to act as fumigants and exerted contact toxicity. However, several oils act as repellents and attractants to disrupt the normal behavior of insects Jayaram *et al*. (2022). Several oils which are derived from *Azadirachta indica, Wedelia prostrata,* and *Solanum torvum* exhibited toxicity against *S. litura* Rengarajan *et al*. (2024). Moreover, EOs could be a safer option due to their low toxicity to mammals and safer for environment.

Although the exact mode of action is not fully understood, understanding how EOs work helps to design stable and effective formulations like nanoemulsions, encapsulations that improve the penetration, persistence, and synergistic effects of oil constituents Isman (2020). In the context of IPM, such as inhibition of acetylcholinesterase (AchE), blocking of octopamine receptors, and disruption of GABA-gated chloride channels in the insect's nervous system, helps to optimize the application strategies, slow resistance development, and ensures compatibility with other control measures Popescu *et al*. (2024). Altaf *et al*. (2024) indicated that the oils from *M. piperita* and *E. camaldulensis* significantly affect the larvae of *Spodoptera frugiperda* and their feeding behavior.

Entomopathogenic fungi, especially species from *Metarhizium* and *Beauveria*, are gaining more attention as potent biocontrol agents for controlling Lepidopteran pests Karthi *et al*. (2024). The *Metarhizium* genus was initially composed of four species, including *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, and *M. taii* Aw and Hue (2017).

Both of EPF and EOs are gaining more attention as eco-friendly alternatives to synthetic pesticides. EOs can act through multiple mechanisms and pose low risk to humans and the environment. Similarly, EPF such as *M. anisopliae* are considered "green pesticides" because they are safer for the environment Sandhu *et al*. (2017). The mode of action of *M. anisopliae* includes, adhesion, spore germination, the formation of appressoria, penetration of the host and ultimately death of the host Peng *et al*. (2022). Their high pathogenic efficiency and biodiversity provide valuable opportunities for developing sustainable biopesticides, reducing reliance on chemical insecticides, and enhancing sustainable agriculture Singh *et al*. (2024).

Despite the increasing interest in botanical extracts and microbial agents as eco-friendly substitutes to synthetic pesticides, their potential against *S. litura* remains insufficiently studied, especially regarding comparative efficacy and chemical characteristics in controlled conditions. Previous research has focused on essential oils and EPF in isolation, with minimal emphasis on their comparative analysis for sustainable pest management. To address this gap, the current study assessed the toxicity of EOs and EPF against *S. litura*, as well as identifying their bioactive components through GC-MS. We propose that the comparative evaluation of these biocontrol agents will demonstrate a significant reduction in pest survival and development. Therefore, the main goal of this study was to check the effect of the insecticidal properties of plant essential oils and *Metarhizium anisopliae* on the larvae of *Spodoptera litura*.

Materials and Methods

Insect culture

Egg clusters and larvae (3^{rd}- 4^{th} instar) of *Spodoptera litura* were collected from Tandla (*Digera arvensis)* weed near College of Agriculture, University of Sargodha. *S. litura* larvae confirmed by its morphological characters. Its body bears light and dark longitudinal bands with paired dorsolateral crescent spots on all segments except the prothorax, most prominent on 1 and 8^{th} abdominal segments. Distinctive dark yellow dorsal strips serve as a key diagnostic feature Thakur *et al.* (2024). This culture was maintained under 23±2 °C temperature, 65 ±5% relative humidity, and 14:10 h L:D photoperiod. The larvae of *S. litura* were placed in Petri plates and Tandla (*Digera arvensis*) tender leaves were provided daily until the larvae transformed into the pre-pupal stage. The pupae then shifted into the plastic jars lined with moist soil for the emergence of adults. The open end of the boxes was covered with muslin cloth. Emerged adults were released in separate cages for mating and egg laying. After the emergence of adults, a 10% Honey-Sugar solution supplemented with vitamin E capsule (10mg/100ml solution) was prepared to feed adults. Egg masses were collected from muslin cloth and the emergence of larvae was observed daily. One generation takes 32 days to complete its lifecycle and the time required to reach F_3 generation was 12-13 weeks. Life cycle duration changes according the temperature and humidity. The F_3 generation was used in further experiments (Figure 1).

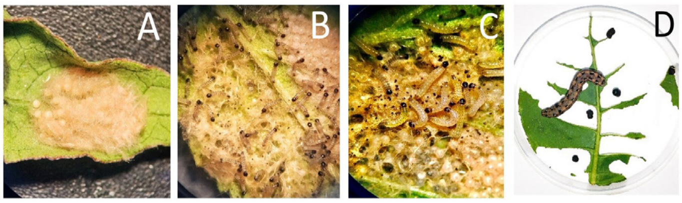

Figure 1: Developmental stages of *Spodoptera litura* under laboratory condition: (A) Egg mass laid on host plant leaf, (B) Newly hatched larvae, (C) Early instar larva showing characteristic clustering and feeding behavior, and (D) Later instar larva feeding on host foliage. Images were taken under stereomicroscopy (B-C) and digital macrophotography (A, D).

Essential oils

To evaluate the bio-efficacy of plant oils against *S. litura*, three oils were used in the experiment: sweet orange (*Citrus sinensis*, Rutaceae), Mint (*Mentha piperita*, Lamiaceae) and lemongrass (*Cymbopogon citratus*, Poaceae). These essential oils (obtained through hydrodistillation process) were obtained from the biocontrol laboratory at the College of Agriculture, University of Sargodha, to test their effectiveness against *S. litura*. Serial dilutions (1.0%, 3.0%, 5.0%) were made of each oil in acetone for bioassay use. Fresh leaves were collected from the field and thoroughly washed with running water, then cut into small pieces. Extraction was done through hydrodistillation method described by Altaf *et al*. (2024). Gas Chromatography-Mass Spectrometry technique was used to profile the individual compounds of EOs. For analysis, GC 7890B, having a DB-5MS fused-silica capillary column, 0.25 µm film thickness, diameter 0.25 mm, and a length of 30m, paired with MS 5977B by Agilent, USA, was used. Previously reported method by (Malik *et al*., 2022) was employed, i.e. the mass spectrum was scanned from m/z 50 to m/z 650 at a rate of 1.5 scans/s, MS source temperature was 230 °C, while the Quadrupole temperature was maintained at 150 °C. The carrier gas used was Helium at a 1 mL/min flow rate. Peaks were identified and labelled automatically by the Mass Hunter software with the NIST library, and quantification was performed based on peak area (%).

Metarhizium anisopliae

M. anisopliae was also taken from the biocontrol laboratory of Entomology, University of Sargodha. The culture was grown on Potato Dextrose Agar (PDA). Fresh culture of *M. anisopliae* was used. Conidia were harvested from 12-day-old culture grown on PDA at 25 ±2 °C. The quality of conidia was assessed using a Neubauer chamber hemocytometer. The conidial suspension was adjusted to different concentrations (10^7, 10^8, 10^9 cfu/ml) in distilled water with 0.05% Tween 80.

Toxicity bioassay for essential oils

Cabbage leaves (*Brassica oleracea*) were collected from outside the college premises. Dip in 0.1% sodium hypochlorite solution and washed with distilled water to remove pesticide residues or microbes. Leaves were allowed to dry completely for 30 min. Next, the leaves were treated with each essential oil concentration separately. Afterward, the treated leaves were used in bioassays. Leaf dip Bioassays were conducted using 3rd instar caterpillar of *S. litura* without insecticide exposure. Larvae of *S. litura* instar were identifies by head capsule width, 3rd instar larvae: 0.68 mm Saljoqi *et al*. (2022).

Collected leaves of cabbage were cut into 9 cm diameter. Leaf discs were dipped into 2 ml of each oil for 10 seconds and dried for 30 minutes. Treated leaves were placed into each Petri Plate (10cm diameter). The larvae were starved for 4 h before the experiment. Then, the larvae were transferred to Petri plates and allowed to feed on treated leaves. Each oil had three concentrations (1.0%, 3.0%, 5.0%), and each concentration had 30 replications, each replication contains 1 larva. In the control treatment, acetone was used alone. Treated larvae were kept in controlled conditions at a temperature of 23±2 °C, with a relative humidity of 65 ±5% and a light/dark cycle of 14:10 h. The mortality data were collected after 72, 96 and 120 h of application.

Toxicity bioassay for Metarhizium anisopliae

The toxicity of *M. anisopliae* was examined by topical application. Three concentrations of *M. anisopliae* were tested (10^7, 10^8, and 10^9 cfu/ml) against *S. litura* larvae. About 4 µl of each concentration was applied on the thorax of *S. litura* larvae by micropipette. Thirty 3rd instar larvae of *S. litura* were used and placed in Petri Plates (lined by filter paper to prevent fungal desiccation). Each treatment repeated three time, each concentration had 30 replicates and one replication contains 1 larva. In control, distilled water was used. Control group treated with sterile water (tween-80 solution in the case of fungal treatment). The mortality data were recorded after 5, 7 and 10 days of application.

Statistical analysis

Corrected mortality was calculated using Abbott's formula (1925). Data were analyzed using a two-factor factorial design under a Completely Randomized Design (CRD), where the factors were *essential oil type* and

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concentration. For the *Metarhizium anisopliae* treatments, a CRD with a single factor (concentration) was used, since only one variable was tested. Percent mortality data were transformed using log transformation prior to statistical analysis, and non-transformed means and standard errors were reported. Means were separated using the Tukey HSD all pairwise comparison test. All the analyses were performed using SPSS 20.1 software.

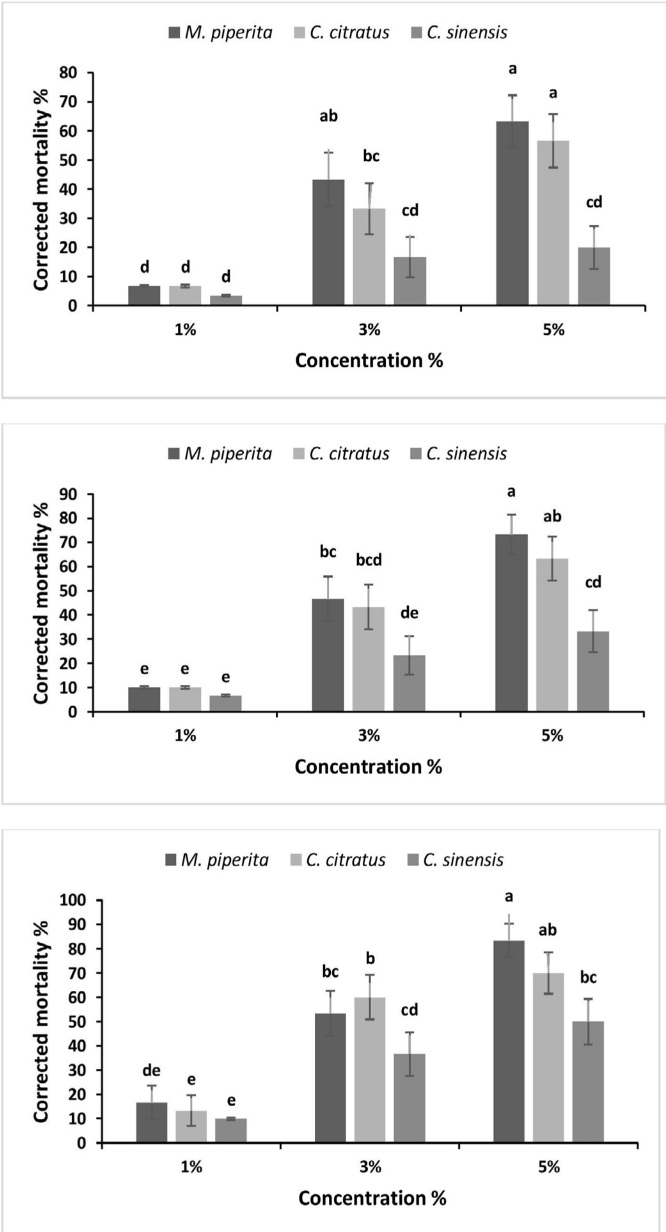

Figure 2: Corrected percent mortality (mean ± SE) of *Spodoptera litura* larvae exposed to different concentrations (1%, 3%, and 5%) of plant-derived essential oils over various time intervals. Columns sharing the same letter are not significantly different at P > 0.05 (Tukey's HSD).

Results

Composition of essential oils

The essential oil (EO) yield from *M. piperita, C. citratus*, and *C. sinensis* was 83.3%, 70%, and 50%, respectively. Gas Chromatography-Mass Spectrometry (GC-MS) analysis revealed the major constituents of each oil. *C. sinensis* contained Apiol (5.59%), Vinyl guaiacol (5.36%), and copaene (4.51%). The principal compounds in *M. piperita* were cubenol (8.43%), trans-carveyl acetate (5.71%), and alpha-Cadinol (5.6%). For *C. citratus* 3,8-Nonadien-2-one, 4,8-dimethyl- (23.66%), Neral (6.22%), and (Z)-2,3-Epoxydecane (5.99%) (Altaf *et al.*, 2024).

Toxicityt of essential oils on Spodoptera litura

A two-way ANOVA showed that both EO type ($F = 4.78$; $P < 0.05$) and concentration ($F = 36.26$; $P < 0.001$) significantly influenced larval mortality. Among the tested EOs, *M. piperita* was most effective, causing 83.3% mortality at a highest concentration (0.5%) after 120 h. *C. citratus* followed with a 70.0% mortality rate under the same conditions (Figure 2). The least effective treatment was *C. sinensis* which caused 50.0% mortality at 5.0% concentration after 120 h of exposure (Figure 2). These findings suggests that *M. piperita EO* exhibits superior insecticidal activity against *S. litura*, likely due to the presence of key bioactive compounds.

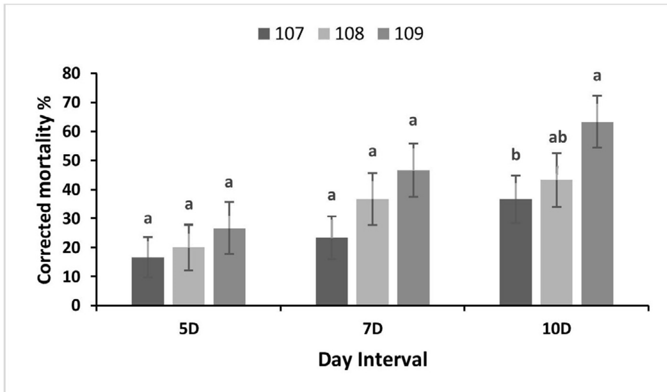

Figure 3: Corrected percent mortality (mean ± SE) of *S. litura* larvae following exposure to three conidial concentrations (10^7, 10^8, and 10^9 CFU/mL) of *Metarhizium anisopliae* over 5, 7, and 10 days. Mortality was assessed under controlled laboratory conditions.

Virulence of Metarhizium anisopliae against Spodoptera litura

The entomopathogenic fungus *Metarhizium anisopliae* also showed insecticidal activity, with mortality varying across time and concentration. No statistically significant differences in mortality were observed at 5and 7 days post-treatment (*P > 0.05*). However, a significant difference ($F = 2.36$, $P < 0.05$) was recorded after 10 days of post exposure. The highest larval mortality rate of 63.3% was recorded at the highest spore concentration (10^9 cfu/ml), followed by 43.3% mortality at 10^8 cfu/ml (Figure 3). This delayed effect is consistent with the known infection dynamics of *M. anisopliae,* where fungal penetration and internal colonization require time to manifest lethal outcomes.

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Discussion

The overreliance on synthetic insecticides has led to serious ecological concerns, including pesticide resistance, environmental contamination, and non-target organism toxicity (Quandahor *et al*., 2024). As a result, there is a growing demand for safer and more sustainable pest management strategies. Among the most promising alternatives are plant-derived essential oils (EOs) and entomopathogenic fungi (EPF), which offer eco-friendly and biologically based methods of pest suppression (Bihal *et al*., 2023).

Essential oils are plant-derived secondary metabolites with demonstrated insecticidal, repellent, and antifeedant properties against various insect life stages (Jyotsna *et al*., 2024). This study evaluated three essential oils (*C. sinensis*, *C. citratus, M. piperita*) and Entomopathogenic fungus *M. anisopliae* against *S. litura* larvae. Our results indicated that all tested essential oils resulted in significant larval mortality, with the most substantial effects noted at higher concentrations and with prolonged exposure times. Among all tested EOs, *M. piperita* showed higher mortality (83.3%) followed by *C. citratus* which caused 70.0% mortality.

The chemical composition of the tested essential oils was previously reported by Altaf *et al*. (2024). GC-MS analyses showed the major compounds were cubenol (8.43%), trans-carveyl acetate (5.71%), and alpha-Cadinol (5.6%) in *M. piperita*. *M. piperita* and its key components showed the toxicity on the survival of *S. litura* larvae. Prasannakumar *et al*. (2023) stated that the *M. piperita* also caused 100% mortality of *Phthorimaea absoluta*. On the other hand, it causes significant average mortality rates of 84.4% and 77.7% when used as nano-biopesticides (Jahan *et al*., 2024) and 100% mortality against *Tribolium castaneum* and *S. oryzae* (Rajkumar *et al*., 2019). These prior findings were consistent with the present study, where *M. piperita* showed greater mortality (83.3%) of *S. litura* and reinforce the broad spectrum efficiency of EOs as promising biopesticides agents.

The effect of essential oils on the tobacco cutworm has been extensively studied (Abdelgaleil and El-Sabrout, 2018). For example, EO of *Artemisia nakaii* exhibited a strong repellent and antifeedant effect on the *S. litura* Liu *et al*. (2021). Comparison to *A. nakaii*, which mainly alters the behavior of insects, but the oils tested in this study produced direct lethal effects, indicating variation in the MoA between different EOs. Such as combined actions increases the capability of essential oils as versatile biocontrol agents.

In favor of the present results, Awad *et al*. (2024) reported that the EOs from *Rosmarinus officinalis* and *Cymbopogon winterianus* affected the development, inhibited the detoxifying enzymes, and caused mortality in *S. littoralis*. This mechanism of action aligns with our observations, *M. piperita* and *C. citratus* caused significant larval mortality, indicating the interference with physiological processes is a common pathway responsible for insecticidal activity of these oils.

The major compounds of *Cymbopogon citratus* were 3,8-Nonadien-2-one, 4,8-dimethyl- (23.66%), Neral (6.22%), and (Z)-2,3-Epoxydecane (5.99%) Altaf *et al*. (2024). Volatile compounds can disrupt insect chemoreceptors, leading to impaired behaviors such as feeding and egg-laying (Qian *et al*., 2024). The volatile compound 3,8-Nonadien-2-one, 4,8-dimethyl-c has been detected in various essential oils recognized for their insect-repelling and toxic characteristics (Altaf *et al*., 2024). While research connecting this compound to *S. litura* is scarce, structurally similar nonadienones have demonstrated both fumigant and contact toxicity against Coleopteran and Lepidopteran pests (Mossa, 2016).

Neral has been reported to inhibit acetylcholinesterase, causing neurotoxin effects that lead to insect paralysis and death (Singh *et al*., 2024). (Z)-2,3-Epoxydecane, an epoxide-containing compound, is less commonly reported in insecticidal studies but is structurally similar to epoxides that are known to interfere with the insect enzyme system. Epoxides can form reactive intermediates that disrupt metabolic pathways and inhibit detoxification enzymes such as glutathione S-transferase and esterases (Ukoroije and Otayor, 2020).

In our study, the essential oil of *C. sinensis* demonstrated moderate insecticidal effects on *S. litura*, with key components identified Apiol (5.59%), Vinyl guaiacol (5.36%), and copaene (4.51%) Altaf *et al*. (2024). Although these findings highlight the potential of EOs, another encouraging option for eco-friendly pest control is the application of entomopathogenic fungi. EPF, like *M. anisopliae*, has been widely identified for its pathogenicity against lepidopteran pests and is considered an effective biocontrol agent. While *C. sinensis* is typically viewed as having less insecticidal effectiveness than other EOs like *M. piperita* or *C. citratus*, these compounds enhance its bioactivity. Citrus-based essential oils show moderate insecticidal and ovicidal properties against Lepidopteran species (Wang *et al*., 2022) and the composition of the oil highly influences their effectiveness. Although *C. sinensis* oil may not be the most potent agent, it possesses potential when combined with other botanical insecticides or as part of an integrated pest management strategy (Khan *et al*., 2024).

C. sinensis plant oil exhibited therapeutic potential in mammals by reducing cancer-induced toxicity via its bioactive compounds Hameed *et al*. (2025), however, in the current research *C. sinensis* demonstrated insecticidal toxicity against *S. litura* larvae, highlighting their broad-spectrum bioefficacy.

However, *M. anisopliae* caused 63.3% larval mortality at 10^9 (cfu/ml) after ten days of application. Entomopathogens are widely used as biocontrol agents to combat various insect pests due to environmental concerns (Bihal *et al*., 2023). This study demonstrates the insecticidal properties of the *M. anisopliae* (63.3%) at 10^9 (cfu/ml) concentration against the 3rd instar larvae of *S. litura*. Kumar *et al*. (2023) describes the virulence of *M. anisopliae* against *S. litura* (Ma-2(2), Ma-2(7) and MaGKS-14 produced > 50% mortality at 96 hrs which was 61.05, 58.79 and 52.11%) and *Aphis craccivora* ($1x10^6$ spores/ml) was 52.64, 69.42, 42.11, 61.58% mortality by Ma-2(2), Ma-2(7), MaGKS-13 and MaGKS-14, respectively. Larval Mortality increased when the concentration of fungus increase (larval mortality rate 63.3% at (109 cfu/ml), followed by 43.3% at 108 cfu/ml), thus, the impact of fungi is directly related to the concentration of conidia. Employing higher concentrations is not difficult since EPFs are safer for both the environment and humans Ghorui *et al*. (2024). Exposure to pathogens affects insects' immunity, which in turn reduces their chances of survival Dhinaut *et al*. (2018).

Our findings are similar to Sharma *et al*. (2024) in which *M. anisopliae* caused 72% and 64% mortality of *S. litura* larvae after 120 h of application and Yusri (2023) observed that the 10^8 conidia/ml caused the highest average larval mortality of *S. frugiperda*, with a mean mortality time of 9.15 days post-application. Our results are supported by Romero-Arenas *et al*. (2014) reported 72.5% larval mortality of *S. frugiperda* using a 53×10^4 con/mL concentration of *M. anisopliae*. Fauzana *et al*. (2020) reported that a concentration of *M. anisopliae* at 50 g/L caused 56% mortality of *O. rhinoceros*. In general, *M. anisopliae* inflicts considerable harm on insect pests through feeding deterrence, a reduced fecundity rate leading to mortality (Zafar *et al*., 2020). Although the mortality rate was not high using EPF, it can be used in combination with other control tactics such as plant essential oils as an alternative to synthetic insecticides. In general, *M. anisopliae* inflicts considerable harm on insect pests through feeding deterrence, a reduced fecundity rate leading to mortality (Zafar *et al*., 2020), and the production of deformed pupae and adults (Kumar *et al*., 2023).

Further research and experiments may focus on isolating the identified key components to determine the effect of each component individually. By focusing on bioactive components and merging synergistic elements, we can improve the active compounds present in Essential oils. Moreover, improved delivery methods like nanoemulsions can boost stability, efficiency, and insecticidal performance. These strategies can significantly enhance the efficiency of Essential oils, making them more competitive with synthetic pesticides.

Conclusion

Use of *M. piperita* oil was more effective in controlling the *S. litura* larvae more compared to other oils tested. Although, *Metarhizium anisopliae* was effective at higher conidial concentrations, its integration with botanical insecticides could enhance larval control efficacy. The efficacy of such oils, along with bioactive components, provides a scientific basis for integrating these agents into integrated pest management programs. The utilization of biopesticides provides a sustainable and environmentally friendly approach to managing insecticide-resistant pests. The findings suggest that EOs can be used as botanical sprays, while EPF can serve as biological control agents, both in combination or individually, to improve sustainability. However, further research is needed to assess the cost-effectiveness, formulation stability, and field-level compatibility of these bioagents under real-world agricultural conditions.

Declarations

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IRB approval

Ethical approval for this study was granted by the Institutional Review Board (IRB) of the University of Sargodha, Sargodha, Pakistan.

Ethical statement

All procedures performed in this study involving human/animal subjects were in accordance with the ethical standards of the institutional research committee.

Generative AI and AI-assisted technology statement

No Generative AI and AI-assisted technologies wer used in the writing process.

Statement of conflict of interest

The authors have declared no conflict of interest.

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