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Larvicidal Activity of *Melissa officinalis* and *Rosmarinus officinalis* Extracts and Their Lethal Impact on Detoxifying Enzymes in *Aedes aegypti* L. Larvae

Mohd Abass Sofi[1], S. Sunitha[1], Mohmmad Ashaq Sofi[2], Anish Mariadhas[3], Jayakumar Manickkam[4], Ezhumalai Parthiban[5], Ramachandran Maduraiveeran[4], Abdolmajid Ghasemian[6]

1. Department of Chemistry, Sathyabama Institute of Science and Technology, Rajiv Gandhi Salai, Chennai- 600119, India
2. Department of Biomedical Engineering, Sathyabama Institute of Science and Technology, Rajiv Gandhi Salai, Chennai-600119, India
3. Department of Mechanical Engineering, Sathyabama Institute of Science and Technology, Rajiv Gandhi Salai, Chennai-600119, India
4. Unit of Applied Entomology, Department of Zoology, University of Madras, Guindy Campus, Chennai -600025, India
5. Department of Zoology, Unit of Aquatic Biodiversity, University of Madras, Guindy Campus, 600025, Chennai, India
6. Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran

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Abstract

Background & Objectives: Mosquito-borne diseases significantly impact global health, particularly in tropical regions. While synthetic insecticides are currently employed to control mosquito vectors, their detrimental effects on ecosystems and persistence necessitate alternative control methods. Botanicals, owing to their diverse phytocompounds, offer potential for controlling and preventing vector-borne diseases by targeting insect eggs and larvae. This study aimed to evaluate the toxicity of *Melissa officinalis* and *Rosmarinus officinalis* extracts (methanolic and aqueous) against *Aedes aegypti* larvae, a vector of arboviruses.

Materials & Methods: Total protein content in control and plant extract-exposed larval homogenates was estimated using bovine serum albumin as a standard. Acetylcholinesterase (AChE) and carboxylesterase assays were performed to determine larvicidal effects. Larval mortality was assessed after 24 hours of exposure.

Results: Our findings revealed that the methanolic leaf extract of *M. officinalis* exhibited superior larvicidal activity (100% at 1000 ppm) compared to the methanolic *R. officinalis* extract (84±2.45% at 1000 ppm). In contrast, the aqueous extracts of both plant species inferred no larvicidal activity. The LC50 and LC90 values for *M. officinalis* methanolic extract were 378.7 ppm and 795.8 ppm, respectively, whereas those for *R. officinalis* were 648.9 ppm and 1152.9 ppm, respectively. Furthermore, biochemical assays measuring total protein, acetylcholinesterase, α-carboxylesterase, and β-carboxylesterase activities were conducted for *M. officinalis*, corroborating and substantiating its larvicidal properties.

Conclusion: This study demonstrates that the methanolic leaf extract of *M. officinalis* possesses significant larvicidal efficacy against *A. aegypti*. These findings suggest that this plant or its phytocompounds could serve as a bioinsecticide, offering a potential alternative to environmentally toxic and non-biodegradable synthetic insecticides.

Keywords: *Aedes aegypti*, Medicinal plants, Bioinsecticide, Acetylcholinesterase, carboxylesterase, Marker enzymes

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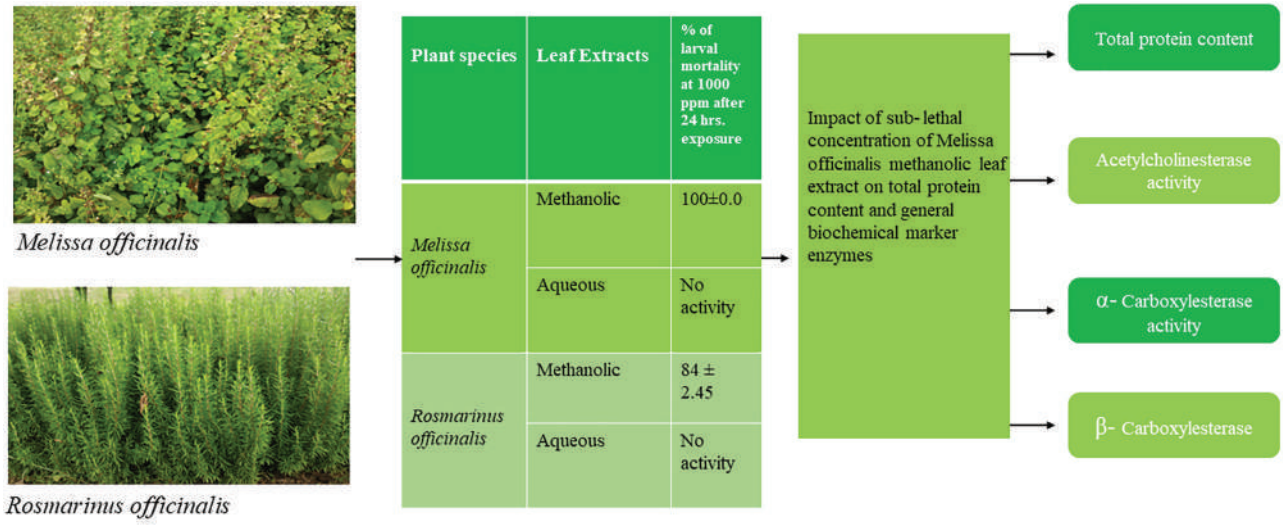
Corresponding Authors: 1. S. Sunitha, Department of Chemistry, Sathyabama Institute of Science and Technology, Rajiv Gandhi Salai, Chennai- 600119, India.
Email: sunithasalla@gmail.com
2. Abdolmajid Ghasemian, Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran. **Email:** majidghasemian86@gmail.com



Graphical Abstract

Introduction

Mosquito-borne diseases pose a growing global health challenge, threatening over 40% of the world's population. By 2050, it is projected that nearly half of the global population will be at risk of arbovirus transmission (1, 2). These diseases not only jeopardize human and animal life but also exert a significant economic impact, particularly in tropical and subtropical regions, which serve as hotspots for such epidemics or pandemics. With approximately 3,500 species spanning 112 genera, mosquitoes represent a diverse and pervasive threat (3). Of particular concern are Aedes mosquitoes, which act as primary vectors for arboviruses responsible for deadly diseases such as Dengue, Chikungunya, and Zika (4, 5). The persistent use of synthetic insecticides and larvicides, however, presents substantial risks to both human health and the environment. These compounds can persist unchanged in ecosystems for extended periods, disrupting ecological balance and infiltrating food chains. Moreover, the interaction between synthetic larvicides and natural biological systems often leads to widespread resistance development (6, 7). It is this growing concern that has catalyzed the exploration and development of economically viable, eco-friendly alternatives, with a particular focus on plant-derived products or phytochemicals as potential insecticides.

Herbal products have a long-standing history in insect control, predating the discovery of synthetic insecticides. Notable examples include nicotine extracted from tobacco leaves, pyrethrums from *Chrysanthemum cinerariaefolium*, and anabasine, an alkaloid derived from a Russian weed. Consequently, phytocompounds or botanicals present a promising alternative to synthetic insecticides and can be integrated into vector control programs, either independently or in combination with other insecticidal agents (8, 9). These plant-based compounds can function as insecticides, targeting mosquito larvae or adults, or serve as repellents to prevent mosquito bites. Such products can be derived from whole plants or specific plant parts through various solvent extraction methods and sophisticated separation and purification techniques (10, 11). *Melissa officinalis* (*M. officinalis*) and *Rosmarinus officinalis* (*R. officinalis*), both members of the *Lamiaceae* family, are medicinal plants native to the Mediterranean region but now cultivated globally. These species are renowned sources of natural bioactive compounds, exhibiting a wide range of pharmacological properties,

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including antibacterial, anti-diabetic, anti-inflammatory, anticancer, and antioxidant effects, among others (12, 13). Numerous plant species from the *Lamiaceae* family have been screened for bioinsecticide development, with various phytoconstituents demonstrating promising insecticidal properties. This has attracted significant research interest towards the development of biodegradable, eco-friendly, cost-effective, and non-hazardous bioinsecticides (14, 15). The primary objective of this study was to evaluate the selective larvicidal efficacy of *M. officinalis* and *R. officinalis* against the fourth instar larvae of *Aedes aegypti* (*A. aegypti*). By focusing on these specific plant species and their potential impact on a key disease vector, this research aims to contribute to the ongoing efforts in developing sustainable and effective mosquito control strategies.

Materials and Methods

Chemicals

Methanol, Fast Blue-B salt, and acetylcholine iodide (AChI) were procured from Hi-Media (Mumbai). α- and β-naphthol, as well as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), were purchased from Sisco Research Laboratories (Mumbai, India). All other chemicals and glassware utilized were of analytical grade.

Collection of plant samples

The plant samples (leaves of *M. officinalis* and *R. officinalis*) were collected during June and July from the higher reaches of the Daksum Anantnag area in Jammu and Kashmir, India, at an altitude of 2,425 meters (Figures 1-3). Identification and authentication were performed at the Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir. The specimens were subsequently deposited in the herbarium under voucher numbers 2833-(KASH) for *M. officinalis* and 2839-(KASH) for *R. officinalis*.

Preparation of extracts

The leaves of *M. officinalis* and *R. officinalis* were shade-dried and ground into a coarse powder using an electric mixer grinder. Subsequently, 10 grams of the powder were extracted in aqueous and methanol solvents separately through decoction and maceration processes, respectively. The extracts were further

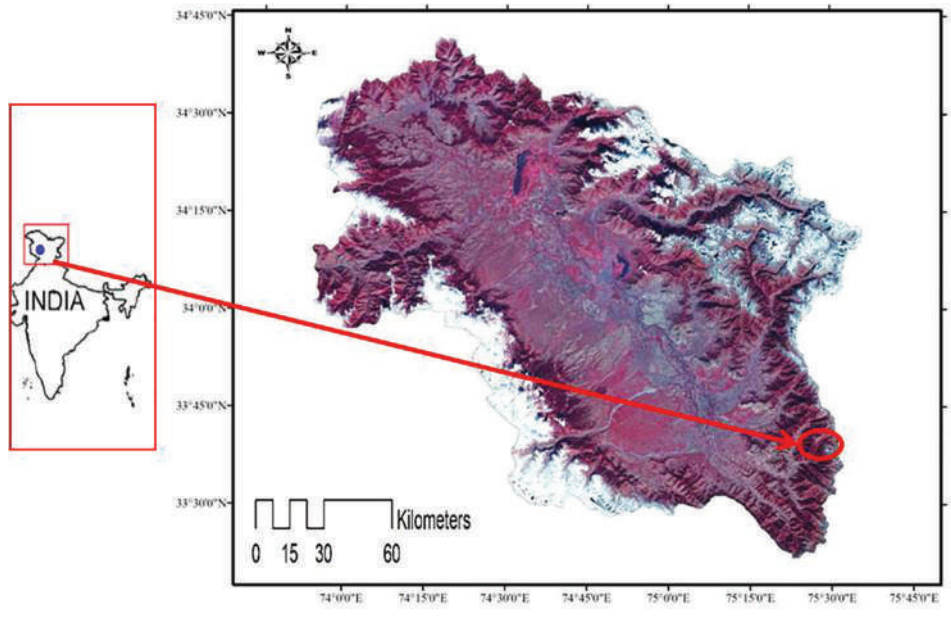

Figure 1. Site of sample collection: Daksum Anantnag (Jammu and Kashmir, India). Latitude: 33°36'43"N. Longitude: 75°26'6"E

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Figure 2. Melissa officinalis

Figure 3. Rosmarinus officinalis

concentrated, solidified using a lyophilizer and a vacuum evaporator under reduced pressure, and preserved in a refrigerator for future use.

Larvicidal bioassay

Mosquito larvae (Figure 4) were collected from residential areas in Chennai, Tamil Nadu (India). Fourth-instar larvae were selected for the bioassay, which was conducted following WHO standards with minor modifications (1996) (16). Ten batches of early fourth-instar larvae were placed in 100 mL of tap water containing each solvent extract at final concentrations of 200, 400, 600, 800, and 1000 ppm. Ordinary tap water with 1% dimethyl sulfoxide (DMSO) served as a control. The larvae were fed a 1:3 ratio of yeast and dog biscuits and maintained at 27±2°C with a 12-hour light/dark photoperiod in the laboratory. After 24 hours of exposure, the percentage of larval mortality was calculated. The experiment was repeated five times to ensure consistency.

Total protein

Following the method described by Lowry et al. (1951), the total protein content in control and plant extract-exposed larval homogenates was estimated using bovine serum albumin as a standard (17).

Acetylcholinesterase assay

The activity of Acetylcholinesterase (AChE) in the larval homogenate was measured using acetylcholine iodide (AChI) as a substrate, following the method of Ellman et al. (1961) (18) with slight modifications by Parthiban et al. (2019) (19). Briefly, 50 µL of larval homogenate was mixed with 800 µL of Na3PO4 buffer (100 mM, pH 7.5), followed by the addition of 50 µL of DTNB (10 mM) and 50 µL of AChI (12.5 mM). The reaction mixture was subsequently incubated at room temperature for 5 minutes, and the optical density was measured at 405 nm.

Carboxylesterase assays

The estimation of α-carboxylesterase and β-carboxylesterase activity from the larval homogenate was quantified following the method of Van Asperen (1962) (20). For the assay, 50 µL of larval homogenate was added to 1 mL of

Figure 4. *Aedes aegypti* larvae

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Na3PO4 buffer (100 mM, pH 7) containing α- and β-naphthyl acetate (250 µM). The reaction mixture was incubated for 30 minutes at room temperature. Subsequently, 400 µL of Fast Blue B salt (0.3%) in 3.3% sodium dodecyl sulfoxide (SDS) was added to each reaction mixture to halt the enzymatic reaction, and the solution was allowed to stand for 15 minutes at room temperature for color development. The optical density was measured using a TSM.EX-200-240V spectrophotometer against a blank at wavelengths of 430 nm and 588 nm for α- and β-carboxylesterase, respectively. The activity of the carboxylesterase enzyme was determined using a standard curve constructed with α- and β-naphthol standards.

Results

Larvicidal activity of *Melissa officinalis* and *Rosmarinus officinalis* extracts against *Aedes aegypti*

In this study, we evaluated the larvicidal activity of *M. officinalis* and *R. officinalis* extracts (methanolic and aqueous) against fourth instar larvae of *A. aegypti*. The methanolic extract of *M. officinalis* exhibited more potent activity compared to that of *R. officinalis*. It is noteworthy that the aqueous extracts of both plants showed no activity under the experimental conditions. After 24 hours of exposure, larval mortality rates of 100% and 84±2.45% were observed at 1000 ppm for *M. officinalis* and *R. officinalis* methanolic extracts, respectively, as shown in Table 1. The lethal concentration (LC) values and other related parameters, including the Fiducial limit, were measured and are presented in Table 1. For the *M. officinalis* methanolic extract, the lethal concentrations (LC50 and LC90) values were determined to be 378.7 ppm and 795.8 ppm, respectively. In comparison, the *R. officinalis* extract yielded LC50 and LC90 values of 648.9 ppm and 1152.9 ppm, respectively.

Mean of five replications±SE; LCL=Lower confidence limit; UCL=Upper confidence limit; LC50=Denotes a lethal concentration at which mortality of exposed larvae is 50%;

Table 1. Larvicidal activity of M. officinalis and Rosmarinus officinalis against fourth instar larvae of Aedes aegypti

SSample ID C	concentration (ppm)	Larval toxicity (%) 24 hrs	LC_{50} (LCL - UCL)	LC_{90} (LCL - UCL)	P value
MM	200	30±3.15	378.7 (300.8 – 440.6)	795.8 (714.4 – 917.8)	1.00
	400	54±2.45			
	600	74±2.45			
	800	86±2.45			
	1000	100±0.0			
	400	26±2.45			
	600	44±2.45			
	800	62±1.99			
	1000	84±2.45			
MA	200	0.0±0.0	-	-	-
	400	0.0±0.0			
	600	0.0±0.0			
	800	0.0±0.0			
	1000	0.0±0.0			
RRA	200	0.0±0.0	-	-	-
	400	0.0±0.0			
	600	0.0±0.0			
	800	0.0±0.0			
	1000	0.0±0.0			

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Table 2. Impact of sub-lethal concentrations of *Melissa officinalis* on proteins and general biochemical marker enzymes

Biochemical Parameters	Control	$LC_{50\ Treated}$
Total Protein (mg/mL of homogenate)	51.45±1.8	35.05±2.8*
Acetylcholinesterase activity (µM of AcT Hydrolysed/min/mg of protein)	380.3±2.8	202.02±1.9***
α-carboxylesterase (mM of α-Naphthol released/min/mg of protein)	1.24±0.0	0.47±0.0**
β-carboxylesterase activity (µM of β-Naphthol released/min/mg of protein)	0.75±0.0	0.32±0.0**

$LC_{90=}$Denotes a lethal concentration at which mortality of exposed larvae is 90%. (-)=absent. MM=*Melissa officinalis* methanol extract, RM=*Rosmarinus officinalis* methanol extract, MA=*Melissa officinalis* aqueous extract, RA=*Rosmarinus officinalis* aqueous extract.

Quantitative analysis of biochemical components

Effect of *Melissa officinalis* methanol extract on total protein level in *Aedes aegypti* larvae

Protein concentration levels were measured in extract-exposed fourth instar larval homogenates and compared against control larval homogenates, as presented in Table 2. The protein levels were found to be 35.05±2.8 µg/µL and 51.45±1.8 µg/µL in extract-exposed and control whole body homogenates, respectively. Statistical analysis revealed that the difference in larval protein concentration levels was significant (P≤0.05) compared to the control.

Acetylcholinesterase activity in *Aedes aegypti* larvae by treating with *Melissa officinalis* methanol extract

Fourth instar larvae treated with *M. officinalis* methanolic extract (24 hours exposure) exhibited a decrease in acetylcholinesterase levels, which was statistically significant (P≤0.05) when compared to the control larval homogenate. After 24 hours of exposure, the acetylcholinesterase level at the lethal concentration was significantly lower than in the control homogenates. The LC50 values for extract-exposed (24 hours) and control homogenates were 202.02±1.9 and 380.3±2.8, respectively, as shown in Table 2. It is important to note that AChE is an enzyme responsible for regulating the activity and level of the neurotransmitter acetylcholine (ACh), which plays a crucial role in the transmission of nerve impulses in neurons. Significant changes in AChE levels can result in impaired neurotransmission, leading to paralysis and death of insect larvae.

Impact of *Melissa officinalis* Methanol Extract on Carboxylesterase Activity in *Aedes aegypti* Larvae

The α- and β-carboxylesterase activities were evaluated in fourth instar larval homogenates exposed to *M. officinalis* methanolic extract for 24 hours and compared to control homogenates. A statistically significant reduction (P≤0.05) in both α- and β-carboxylesterase levels and activities was observed in extract-exposed homogenates relative to the control, as illustrated in Table 2. This decrease in carboxylesterase activity is evidenced by the diminished release of α- and β-naphthol, respectively. In extract-exposed larvae, the α-naphthol release level was recorded at 0.47±0.0 µM/min/mg, compared to 1.24±0.0 µM/min/mg in the control. Similarly, the β-naphthol release level in extract-exposed larvae was 0.32±0.0 mM/min/mg, while in the control, it was 0.75±0.0 mM/min/mg of protein.

The value represents the mean±SD of three replicates using samples from different preparations. NS indicates not statistically; significant difference between the control and experiment (P<0.05*).

Discussion

Plants produce a diverse array of primary and secondary metabolites that serve as natural defenses against harmful microbes and predatory insects, making them excellent candidates for anti-*A. aegypti* therapies (21). Natural products

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have been extensively employed as insecticides and larvicides in numerous trials aimed at combating insect-borne diseases, particularly those transmitted by Aedes mosquitoes (22). In recent years, plant extracts and their phytochemicals have garnered significant attention as potential vector control agents (23). A wide spectrum of plant extracts and their diverse phytocompounds have been evaluated and screened in the quest for novel, effective insecticides and larvicides (24, 25).

Essential oils derived from plants and their extracts have been demonstrated in various research studies to function as effective insect repellents, possessing both insecticidal and larvicidal properties. Importantly, these natural compounds typically pose limited toxicity risks and have negligible environmental impacts (26). In our study, we observed 100% larval mortality at 1000 ppm for the methanolic extract of *M. officinalis*. In contrast, *R. officinalis* exhibited 84±2.45% larval mortality at the same concentration after 24 hours of exposure. It is noteworthy that no activity was observed in the aqueous extracts of either plant. The methanolic extract of *M. officinalis* demonstrated markedly stronger larvicidal activity compared to that of *R. officinalis*. Consequently, we focused our subsequent investigations on the effects of *M. officinalis* methanolic extract on the detoxifying enzymes of *A. aegypti* larvae. To develop more effective insecticides, it is crucial to elucidate how botanical compounds influence the physiology of target insects. In this study, we examined the systemic effects of *M. officinalis* methanolic extract on fourth-instar larvae after 24 hours of exposure, with particular emphasis on alterations in *A. aegypti* biochemical parameters at specific LC50 levels.

Proteins play a crucial role in cuticle development and insect metamorphosis, being essential for growth and development. During ecdysis (the moulting period), proteins contribute to the formation of a new cuticle by replacing the old cuticle covering (27). The synthesis of chitin is also of immense importance to all arthropods, enhancing the digestion process and protecting the brush border from external pathogens or toxins (28). When protein expression is down-regulated due to xenobiotic invasions, the growth and development of insects are significantly affected (29). Therefore, it is imperative to investigate how plant-derived metabolites influence protein expression in insects, with the aim of formulating and proposing novel, efficacious metabolites for pest control or management. In light of this, numerous researchers have screened active metabolites from various plants that directly or indirectly affect protein expression in diverse insects, including mosquitoes, while also quantitatively and qualitatively analyzing important insecticide-resistant biomarker enzymes. As previously discussed, proteins play a vital role in insect metamorphosis, being involved in chitin synthesis and cuticle formation. When insects are exposed to plant-based biopesticides, there is often a dysregulation (either upregulation or downregulation) of protein function (29, 30). In our study, the protein content in larval homogenates subjected to plant extract was significantly lower than in control larvae. These results align with previous studies (30-32), where protein levels decreased upon exposure to plant extracts at sub-lethal concentrations. Consequently, this prior research evidence provides a plausible explanation for our observation of decreased protein synthesis in whole-body homogenates of *A. aegypti* larvae exposed to plant extract.

Acetylcholine, a neurotransmitter responsible for transmitting nerve impulses across the synaptic cleft of neurons, is released into the junction gap (synaptic cleft) when a nerve impulse reaches the presynaptic neuron. It stimulates ligand-gated sodium channels in the postsynaptic membrane or axons, facilitating the movement of sodium ions through the

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postsynaptic membrane. This action generates an action potential that is transmitted across the neuron as a wave, known as a nerve impulse (33). Following this action, during the resting phase, acetylcholine is hydrolyzed into acetate and choline by the enzyme AChE. The byproducts of this enzymatic reaction are then reuptaken by the presynaptic neuron, repeating the cycle (34, 35). Our study revealed a decrease in the overall level of AChE upon exposure of A. aegypti fourth-instar larvae to methanolic *M. officinalis* extract. This reduction in AChE levels results in the disruption of nerve impulse transmission, ultimately leading to paralysis or death of the insects (33, 36). The enzymes α- and β-Carboxylesterase are involved in detoxifying a wide range of allelochemicals to which insects are exposed (19). Carboxylesterase (CarE α & CarE β) is one of the most significant detoxifying enzymes in various insect pests, particularly in mosquitoes. Esterases are the primary mechanisms behind resistance to carbamates, pyrethroids, and organophosphates. It has been demonstrated that the up-regulation of esterase levels in various insect pests, including mosquitoes, is related to their resistance against insecticides (37, 38). Previous research investigating isozyme expression levels against the 4th instar larvae of *A. aegypti* using a methanolic extract from Piper nigrum found that the extract significantly inhibited esterase expression. Similarly, Sofi et al. (2022) (30) recently investigated the effect of methanolic extract from Artemisia absinthium on the CarE (α & β) expression level in *A. aegypti* and found that the tested extract successfully inhibited enzyme production. In our study, when *A. aegypti* fourth instar larvae were exposed to plant extract for 24 hours, the activity of α- and β-Carboxylesterase enzymes was reduced due to the downregulation of these enzymes, as depicted in Table 2. This finding indicates that the methanolic extract of *M. officinalis* can inhibit the specific enzyme activity of the tested organism without developing resistance. Consequently, our findings strongly support the development of novel insecticides against a wide range of insect pests, including mosquitoes, based on the inhibition of acetylcholinesterase and α- and β-carboxylesterases by *M. officinalis* leaf extract.

Conclusion

This study has demonstrated that *M. officinalis* leaf extracts are effective in eradicating *A. aegypti* larvae and inhibiting specific detoxifying enzymes, namely AChE and α- and β-carboxylesterases. To further elucidate the mechanisms underlying these effects, it is recommended that a comprehensive phytochemical analysis be conducted on the leaf extract to identify the compounds responsible for its larvicidal properties. The extracts or phytocompounds obtained from *M. officinalis* have potential applications in treating stagnant water bodies that serve as breeding grounds for mosquitoes and other insects. Before recommending this plant for inclusion in mosquito vector control programs, further extensive research is imperative. This should encompass in-depth phytochemical profiling to develop novel bioinsecticide formulations, field testing to assess real-world efficacy, and investigations into the precise mode of action of the active compounds. The use of plants as a source of mosquito larvicides may offer significant advantages, as they are generally biocompatible and non-hazardous. By employing these botanicals in mosquito control strategies, it may be possible to minimize costs and reduce environmental pollution compared to synthetic larvicides.

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Conflict of Interest

The authors declare no conflicts of interest.

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Ethics Approval and Consent to Participate

Not applicable.

Code of Ethics

This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Consent for Publication

All authors have consented to the publication of this manuscript.

Availability of Data and Materials

The datasets used and/or analyzed during the current study will be made available by the corresponding author upon reasonable request.

Funding

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Authors' Contribution

M.A.S, S.S, M.A.S, A.M, and J.M. performed the experimental work and wrote the main manuscript text. E.P, R.M, and A.G. edited the manuscript and provided final approval of its scientific content.

List of Abbreviations

LC: Lethal concentration, AchI: Acetylcholine iodide, DTNB: (5-5-dithiobis 2-nitro benzoic acid, CBT: Centre for biodiversity and taxonomy: DMSO: Dimethyl sulfoxide, AChE: Acetylcholinesterase, RT: Room temperature, SDS: sodium dodecyl sulfoxide, OD: Optical density, BSA: Bovine serum albumin, TSM: Thermo Scientific Multiskan, ACh: acetylcholine, CarE: Carboxylesterase

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