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Plant-Based Bioactive Compounds as Sustainable Alternatives for Multi-Stage Control of Malaria Vector Anopheles Stephensi

Iqra Batool[1], Ghulam Ali Bugti[2](Corresponding Author)**, Arif Ali[3], Abdul Hafeez Mastoi[4], Hamid Ibrahim[5], Fatima[6]**

[1] Department of Entomology, Lasbela University of Agriculture Water & Marine Science, Uthal, Balochistan, Pakistan, iqrahanbhi786@gmail.com, https://orcid.org/0009-0009-9949-6264
[2], Affiliation Same as Above, gabugti@outlook.com, https://orcid.org/0000-0001-8266-6698
[3], Affiliation Same as Above, arifalirao.agri@luawms.edu.pk, https://orcid.org/0009-0003-6859-8184
[4], Affiliation Same as Above, hafeez.agri@luawms.edu.pk, https://orcid.org/0009-0002-2626-0495
[5], Affiliation Same as Above, hamidibrahimazad@gmail.com, https://orcid.org/0009- 0006-1263-4117
[6], Affiliation Same as Above, fatimarasoolbakhsh@gmail.com, https://orcid.org/0009-0009-4824-9036

Abstract

The increasing resistance of Anopheles stephensi to conventional insecticides has created an urgent need for safe, eco-friendly alternatives for mosquito control. This study evaluated the larvicidal, ovicidal, and adult repellency effects of four botanical extracts: Neem (*Azadirachta indica*), Citrus (*Citrus sinensis*), Moringa (*Moringa oleifera*), and Nerium (*Nerium oleander*) at concentrations of 30%, 60%, and 90% against Anopheles stephensi under laboratory conditions. Bioassays were conducted using standard WHO protocols, and data were analysed through Univariate ANOVA and post-hoc tests to determine the effect of treatment and concentration. Results showed that both treatment and concentration had highly significant effects ($p < 0.001$) on larval mortality, egg hatchability, and adult repellency, while the treatment concentration showed a consistent dose-dependent pattern among all extracts. Our results showed that the Neem extract exhibited the strongest adult repellency at 90% concentration compared to other concentrations (60.00 ± 1.33%), followed by Citrus (53.33 ± 1.33%). Moringa (23.33 ± 1.33%) and Nerium (26.67 ± 1.33%) had lower repellency. The minimum LC^{50} for adult repellency was observed in Neem (74.2), and an LC50 of 83.6 was noted in Citrus treatment. However, no significant results were observed in Nerium and Moring treatments, respectively. Whereas, Neem extract exhibited the highest larvicidal efficacy with a maximum response of (68.89±4.33%), followed by Nerium (58.89±4.33%), Citrus (51.11±4.33%), and Moringa (40.00±4.33%). While the minimum LC^{50} value was (53.6) in Neem treatment, it was (55.1) in Citrus treatment, and the maximum LC^{50} value was (61.3) and (64.8) in Nerium and Moringa treatment against larvae, respectively. In ovicidal assays, Citrus and Neem extracts most effectively reduced egg hatchability, while in repellency tests, Neem (41.11±2.07%) and Citrus (37.78±2.07%) produced the strongest deterrent effects, particularly at 90 concentration. Furthermore, the minimum LC^{50} for adult repellency was observed in Neem (74.2), and an LC^{50} of 83.6 was noted in Citrus treatment. However, no significant results were observed in Nerium and Moring treatments, respectively. Results showed that the role of botanical bioactive compounds in disrupting mosquito development and behaviour supports their use as sustainable components in Integrated Vector Management (IVM) programs.

Keywords: Anopheles stephensi, botanical extracts, Neem, Citrus, larvicidal activity, ovicidal effect, repellency, eco-friendly vector control.

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Introduction

Mosquitoes (*Diptera: Culicidae*) comprise more than 3,600 species globally and remain among the most medically important insect groups due to their ability to transmit pathogens causing malaria, dengue, yellow fever, and filariasis. (Burdick, 2015). Anopheles stephensi is a primary malaria vector in South Asia and the Middle East, and was first recognised as a malaria-transmitting species by Tadesse et al. (2021). Globally, more than 465 Anopheles species have been identified, of which approximately 70 are competent malaria vectors. (Sinka et al., 2012). Among those, around 23 species of Anopheles have also been reported in Pakistan. In which 2 Anopheles species have been confirmed as vectors of malaria fever, i.e. Anopheles culicifacies as the key causal agent of malaria in rural areas, and Anopheles stephensi is the principal malaria vector in the urban regions of the country.(Ilahi and Suleman, 2013; Jahan and Hussain, 2011) In many regions where malaria is prevalent, it is becoming more and more difficult to control due to the failure of vector control measures and the use of anti-malarial medications. In 91countries of the world, the fever of malaria is endemic, with about 40% of the world's public is in threat. Resistance of the parasite (Delnat et al., 2020)

The control vector of mosquitoes, varieties of inorganic repelling substances and along with chemicals, namely Malathion, DDT, organophosphate, organochlorine, deltamethrin and synthetic pyrethroid pesticides are utilised(Delnat et al., 2019). But their poisonous and hazardous substances, these kinds of pesticides may pose a threat to the environment and other unintended organisms, such as people, animals and also, mosquitoes are likely to acquire resistance against these medications in the near future. (Bansal et al., 2014). The use of these synthetic pesticides is that these pose a threat to non-target organisms and may be hazardous to other components of the ecosystem.(Ohia et al., 2013)

Considerable initiatives have been implemented to control mosquitoes more than any other insect of medical importance. Botanical substances have been used as a traditional method by human societies in various parts of the globe towards causal agent & the other species of insects. Plants may be an excellent source of alternative compounds that can serve as alternative agents for mosquito management.(Baranitharan and Dhanasekaran, 2014). Phytochemicals are also good sources; they can perform as larvicidal, insect growth regulator and adult repellent. (Krishnappa et al., 2012).

Among the plants, Neem, which belongs to the genus Azadirachta indica (Family: Meliaceae), showed advantageous characteristics in public health, and its derived materials have displayed insecticidal actions (Aguirre et al., 2024). A. indica products containing limonoids have been examined against different categories of mosquito and neem is a member of the best replacement for suppression of mosquito declared on a limonoid, gedunin from A. indica, showed hundred percent at 50 and 10 ppm, when estimated for its lethal activity against the Aedes aegypti fourth instar larval stage. (Aguirre et al., 2024). Poisonous impacts of essential oil and leaves of Nerium oleander were also reported to possess an expel impact upon the Culex pipiens (Baz et al., 2024). The Nerium oleander leaves ethanolic extract shows insecticidal action towards Drosophila rufa as well as Trogoderma granarium and declares to hold antifeedant and pesticidal action against diamondback moth (Plutella xylostella. (Raveen et al., 2017). The larvicide actions of fluidy seed, extract of Moringa oleifera, upon the larval stage of Anopheles gambiae in Nigeria, are the primary key vector of malaria, and to research its effects on mosquitoes, a fish is an ordinary non-target organism present in the mosquito's natural environment(Alves et al., 2020). Moringa is often referred to as a "miracle tree" because it contains a distinctive array of bioactive compounds known as glucosinolates and isothiocyanates. These compounds have been shown to possess antihypertensive, antineoplastic, and antibacterial properties. (Ohia et al., 2013). The plant of Citrus leaves active ingredients extract lemon is known to hold fragrant and medicinal significance of insect-repelling substances(Adeniyi-Akee et al., 2025). The lemon leaves are considered safe, and they are utilised in the preparation of juice beverages and green tea because of their therapeutic properties (Amusan et al., 2005; Giatropoulos et al., 2012). In ancient medicine, Lemon citrus (Citrus lemon Burm) has long been traditionally used as a natural insect repellent in Iran (Sanei-Dehkordi et al., 2016).

Malaria is a deadly parasitic illness spread to humans through the bite of an Anopheles stephensi mosquito carrying the infection. In Pakistan, A. stephensi and A. culicifacies are the dominant species responsible for malaria transmission. Malaria fever is endemic in the year 2022 between January and August. There were over 3.4 million probable cases confirmed cases arise in August 2021 22, and also 41,368 cases reported in 2022 August.(directorate-of-malaria-control, 2023). Manzoor et al. (2020) reported malaria as a major public health burden in the country, with millions of suspected cases reported annually, especially following major flooding events (directorate-of-malaria-control, 2023)

Considering the aforementioned claim, it was found that several plant species have the capacity to suppress mosquitoes. Consequently, in this research, we analysed the extracts from Azadiracta indica, Moringa oleifera, citrus lemon, and Nerium oleander leaves on the malaria-causing agent Anopheles stephensi. The objective of the current study is to evaluate the adulticidal, ovicidal, and repellent properties of plant extracts of four plant species from the Lasbela District of Balochistan and record their efficacy against Anopheles stephensi mosquitoes.

Materials and methods

Plant Collection and Identification

The plant materials used in this study were selected based on their documented bioactivity and ethnobotanical significance in mosquito control. Fresh and healthy leaves of Azadirachta indica, Moringa oleifera, Citrus limon, and Nerium oleander were collected from various locations of the Lasbela University of Agriculture, Water and Marine Science, Uthal, Balochistan. For identification, the specimens were prepared, tagged, and deposited in the Department of Horticulture (LUAWMS), Uthal, Balochistan. Collection occurred during the plants' peak vegetative phases to ensure optimal concentrations of bioactive compounds. Harvesting was performed in the early morning to avoid the loss of volatile constituents, especially in Citrus limon and A. indica. Each plant species was taxonomically identified by expert botanists using standard floras and field keys.

Plant Extraction procedure

Before processing, the collected materials were cleaned using distilled water to get rid of dirt, debris, and possible pesticide residues. Plant samples were then shade-dried at ambient moderate temperature (26 ± 2°C) for 7–10 days to prevent degradation of heat-sensitive phytochemicals, following the protocol used by (Murugan et al., 2012). Plant extract (500ml) was obtained using an electrical juicer from collected leaves (1000 g) of each plant species. From this stock solution, different concentrations, likewise 30%, 60% and 90% by dissolving the plant extracts in distilled water, were formulated, and these concentrations were used as adult repellent larvicidal, and ovicidal.

Mosquito Collection and Rearing

Adult Anopheles stephensi mosquitoes were gathered from Lasbela District locations and cultured in the Laboratory, Department of Entomology, LUAWMS, Uthal. Using standard vector surveillance techniques, and subsequently identified based on morphological characteristics using established taxonomic keys (Das et al., 1990). Post-identification, adults were sexed and housed in insectary cages under conditions (27 ± 5°C, 70–80% RH, and 12:12 h L: D photoperiod), following WHO recommendations.

For colony establishment, adult mosquitoes were maintained in a 30 × 30 × 30 cm rearing container made of plastic were continuously supplied with a 10% sucrose solution via cotton wicks as an energy source. (Rajkumar and Jebanesan, 2007). Gravid females were provided a blood meal within 24 hours using freshly thawed chicken liver as a substitute blood source to stimulate oogenesis and egg maturation, as the use of live animals was restricted due to ethical considerations. Oviposition was induced by introducing moist filter papers and small water bowls as egg-laying substrates within the cages. Collected eggs were transferred to enamel or plastic trays filled with dechlorinated tap water to allow hatching. The mosquito larvae were cultured in these trays and fed a standardised larval diet composed of a 3:1 mixture of powdered dog biscuits (e.g., Pedigree®) and yeast powder to ensure adequate protein and carbohydrate content for optimal larval development. Larval trays were cleaned regularly to prevent fungal growth and maintain water quality. Pupae were collected using pipettes and transferred to small cups placed within adult emergence cages. Emerged adults were allowed to mate freely in the cages and were maintained for further experimental or colony maintenance purposes. After that, the Pupae were picked up from the trays and placed in a cup holding drinking water and kept in the 45 × 45 × 40 cm insectary, where the adults came out. The adults were housed in glass cages and fed a 10% sucrose solution in a jar with a cotton wick constantly. The adults were fed a blood meal made from chicken liver on day five. For oviposition, glass Petri plates lined with filter paper and containing 50 mL of distilled water were placed within the cages.

Adult Repellency bioassay test

The mosquito repellency was performed in laboratory conditions as mentioned earlier. The blood feeders, 3 to 4 days old, 10 female mosquitoes were placed into a 45 × 30 × 45 cm cage made of net. Adult repellency was evaluated by exposing 200 adult mosquitoes to cotton pads soaked with botanical extracts at 30%, 60%, and 90% concentrations to find the adult repellency % of adults and compare with the control groups. Repellency data were observed 48 hours later. Each treatment was replicated three times.

Larvicidal Bioassay test.

The larvicidal bioassay of all treatments was performed with concentrations of 30%, 60% and 90%. The required test concentrations and quantities of test solutions were obtained to execute the bioassay test. The larval culture was maintained for the experiment. Twenty larvae of third-instar from lab culture were placed 50 ml plastic cup with the appropriate test dosage along-with untreated control group. The mortality data were noted after 48 hours. Each treatment had three concentration and each concentration was replicated three times.

Ovicidal Bioassay test

The ovicidal bioassay was carried out in compliance with the instructions of (Reegan et al., 2015) 10 eggs of A.stephensi were placed in a test container filled with different concentrations of plant extracts likewise 30%, 60% and 90% and a control treatment filled with the same amount of distilled water. Data was observed after 48 hours of exposure to the treatment the no of hatched and unhatched eggs was determined. All treatment concentration replicated three times. And the data was recorded after 48 hours.

Data analysis. Larval mortality and egg non-hatchability response percentages were calculated by $\text{lmr\&emr} = \frac{\text{Mortality \%}}{\text{Total number of Inects}} \times 100$

Further data was analysed using one-way (ANOVA), and the effects of different treatments were compared using Tukey's test to obtain the level of significance among various concentrations on different life stages of the mosquito. All analyses were done using the computer software SPSS version 21 (Crop, 2012) at a significance level of $P \leq 0.05$

Results

Botanical extracts have gained significant attention as eco-friendly alternatives for mosquito control due to their biodegradability, low toxicity to non-target organisms, and effectiveness against various mosquito species. These natural plant-derived compounds contain bioactive substances such as alkaloids, terpenoids, phenolics and flavonoids that exhibit ovicidal, larvicidal, pupicidal, and adult repellency properties. Extracts from plants like Moringa oleifera (leaves and seeds), Citrus limon (fruit peels), Nerium oleander (leaves), and Azadirachta indica (leaves and seeds) have demonstrated strong insecticidal effects on all life stages of mosquitoes. Unlike synthetic insecticides, botanical extracts reduce the risk of resistance development in mosquito populations and minimise environmental contamination. The results of our experiment explained the possible control of different life stages of mosquitoes in detail.

Ovicidal response

The highest concentration of plant extract had a significant impact on egg hatchability, as evidenced by the maximum egg non-hatchability at 90% concentration on citrus extract, which showed (56.67 ± 1.6%) efficiency, followed by Neem (46.33 ± 1.6%). Conversely, lower mortality was recorded for Nerium (14.67 ± 1.6%) and Moringa (17.33 ± 1.6%) prospectively. Whereas the lowest LC^{50} (84.7) was observed in citrus. However, Neem also showed a good result with an LC^{50} value of 96.2. The maximum LC^{50} was observed in Nerium and Moringa treatment, shown in Table 1. Overall treatment effects were highly significant ($Df=4$, $F=26$, $P < 0.001$). Figure 1.

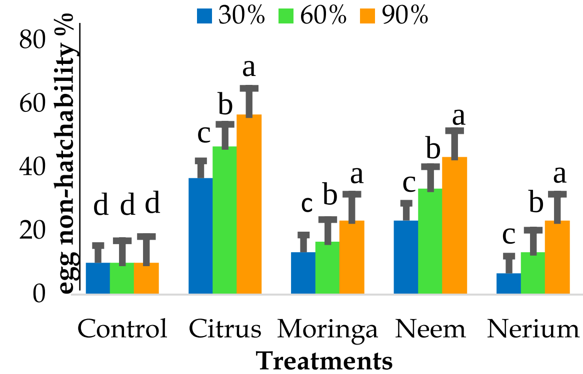

Figure 1. Effect of different botanical extracts (concentrations) on egg non-hatchability. A stephensi.
Figure showing the same letter on Bars are not significantly different (P < 0.05).

Larvicidal response

The larval mortality across the different plant extract concentrations examined is depicted in Figure 2. The highest larval mortality was 68.88 ± 2.13% at the highest dose of 90% neem extract, followed by citrus (58.52 ± 2.20%), nerium (53.29 ± 1.30%), and moringa (40.60 ± 3.33%). The calculated LC_{50} Showed minimum (53.6) value in Neem treatment, while the LC_{50} (55.1) was noted on Citrus treatment. Whereas, the maximum LC_{50} (61.3) and (64.8) were observed in Nerium and in Moringa treatment, respectively. Table 1. Both concentration and botanical therapy had a substantial impact on larval mortality, according to statistical parameters. A significant impact was shown by concentration (Df=4, F = 10.4, P = 0.001). In contrast, the control treatment had the lowest death rate (10 ± 1.30%). Figure 2.

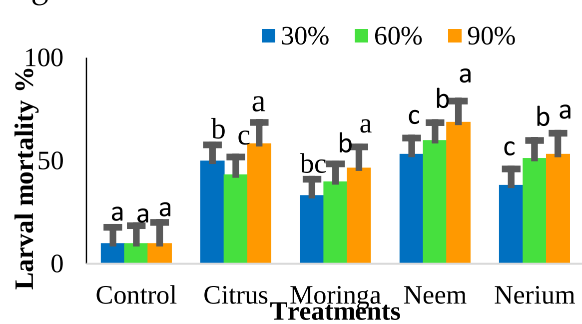

Figure 2. Effects of Different Botanical Extract Concentrations on the Larval Mortality of A. stephensi. Figure showing the same letter on Bars are not significantly different (P < 0.05).

Adult repellency response

The findings demonstrated that adult repellency is significantly impacted by plant extracts. Neem extract showed the strongest repellency at 90% concentration compared to other concentrations (60.00 ± 1.33%), followed by citrus (53.33 ± 1.33%). Moringa (23.33 ± 1.33%) and Nerium (26.67 ± 1.33%) had lower repellency values. The minimum LC_{50} for adult repellency was observed in Neem (74.2), and the LC_{50} (83.6) was noted in Citrus treatment. Whereas no significant result was observed in Nerium and in Moring treatment. Table1. The efficiency of adult repellency was also confirmed by the analysis of variance (Df=4, F=49.3, P <0.001). Repellency was lowest in the control group (10.00 ± 0.00%). Figure 3.

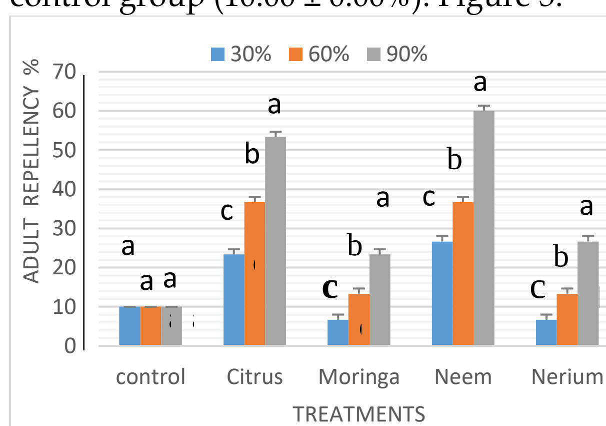

Figure 3. Effects of Different Botanical Extract Concentrations on Adult Repellency of A. stephensi. Figure showing the same letter on Bars are not significantly different (P < 0.05).

Table 1. Lethal Concentration Dosage LC_{50} of different plant extracts against A. stephensi

Treatments	Bioassay Type	LC(50)/	Slope	95% Confid	Remarks

		RC (%)	± SE	ence Limits	
Neem	Larvicidal	53.6	2.34 ± 0.42	44.8 – 64.1	Good fit
	Ovicidal	96.2	1.28 ± 0.36	72.5 – 168.4	Wide CL
	Repellency	74.2	2.11 ± 0.38	61.5 – 89.3	Good fit
Citrus	Larvicidal	55.1	2.08 ± 0.47	44.1 – 68.9	Good fit
	Ovicidal	84.7	1.67 ± 0.40	65.3 – 118.6	Moderate fit
	Repellency	83.6	1.89 ± 0.41	68.2 – 102.4	Good fit
Nerium	Larvicidal	61.3	1.95 ± 0.52	47.2 – 86.5	Moderate fit
	Ovicidal	>150	—	Not reliable	Low response
	Repellency	>120	—	Not reliable	Low response
Moringa	Larvicidal	64.8	1.76 ± 0.49	50.3 – 92.7	Moderate fit
	Ovicidal	>150	—	Not reliable	Low response
	Repellency	>120	—	Not reliable	Low response

The effect of plant extract on 50% mortality of insects was analysed by a regression-probit, and the data were further manually calculated to obtain LC_{50} values.

Discussion

This study was conducted to evaluate the larvicidal, ovicidal, and adult repellent activities of four botanical extracts: Neem (Azadirachta indica), Citrus (Citrus sinensis), Nerium (Nerium oleander), and Moringa (Moringa oleifera) against Anopheles stephensi, a principal malaria vector in South Asia. Three concentrations (30%, 60%, and 90%) were tested to assess dose-dependent responses in mortality, egg hatchability, and adult repellency. Results from the Univariate ANOVA analyses demonstrated that both treatment type and concentration had highly significant effects ($p < 0.001$) on all three bioassay tests.

In the larvicidal response, Neem extract exhibited the highest mortality (68.89±4.33%), followed by Nerium (58.89±4.33%), Citrus (51.11±4.33%), and Moringa (40.00±4.33%). Increasing concentrations led to higher larval mortality, confirming a strong dose-response relationship. Our research is in excellent agreement with studies (Alayo et al., 2015) that assess the repellent impact of an extract formulated in an aqueous cream base against Anopheles gambiae mosquitoes and examine the larvicidal activities of extracts of Cassia mimosoides leaves and pods as a possible agent in vector control of malaria. Anopheles gambiae larvae in their third and fourth instars were used to test petroleum spirit, ethanol, water, and dichloromethane extracts. N-N-diethyl-m-toluamide (DEET), he found that the extracts' mortality rate showed a dose-related response, with 100% mortality in 2 mg/ml petroleum ether and dichloromethane extracts. Demouche (2023) demonstrated Methanolic extracts of Coprinopsis cinerea extract observed a dose dependent response and good larvicidal and pupicidal mortality with LC(50) and

LC(90) values of 1.10 and 4.37 mg/mL against pupae, 24 hours post treatment, and 1.26 and 2.35 mg/mL against the fourth instar larvae, respectively, when tested for insecticidal activity at concentrations of 0.62, 1.25, 2.50, 3.75, and 5 mg/mL. In a concentration-dependent way, C. cinerea extract decreased the activity of the enzyme acetylcholinesterase (AChE). The inhibition percentages obtained 48 hours after treatment were 35.11 +/- 7.44 and 51.83 +/- 4.04% for the pupal stage and 30.98 +/- 2.97% and 48.77 +/- 4.72% for the larvae in their fourth instar.

We observed different control levels for all plant extracts, as we observed ovicidal response; both treatment and concentration significantly influenced egg hatchability. Citrus and Neem extracts showed the strongest ovicidal activity, with mean hatchability rates of (46.67±1.84%) and (33.33±1.84%), respectively, whereas maximum egg hatchability was found in Moringa and Nerium treatment. These results highlighted the ability of plant-derived compounds to inhibit egg viability and embryonic development. Similar observations were documented by (Alves et al., 2020) and found the effect of Myracrodruon urundeuva bark MuBL, heartwood MuHL and Moringa oleifera seeds as larvicidal agents against Aedes aegypti; MuBL and MuHL were ovicidal agents with EC(50) of 0.26 and 0.80 mg/mL (260 and 800 ppm), respectively. Egg surfaces showed significant distortion and degradation after 48 and 72 hours of incubation.

Neem and Citrus performed the best adult repellency percentages(60.11±2.07%) and (53.28±2.07%), respectively. Repellency increased markedly with higher concentrations, particularly at 90 concentrations. Moringa and Nerium exhibited weaker repellency, indicating less potent volatile compounds. Neem consistently showed the strongest multi-stage bioactivity, followed closely by Citrus, while Moringa and Nerium were comparatively less effective. The adult repellency in neem extract is also due to (Aguirre et al., 2024; Chatterjee et al., 2023) Neem tree (Azadirachta indica) metabolites have biological activity against various biological parameters such as lifespan, fecundity, fertility, and transgenerational survival from larvae to adults, and suggest adult survival patterns. The F1 generation survival was significantly impacted at doses of 100 ppm and 1,000 ppm. They also observed that those that consumed the nanoformulation showed a trend of decreased oviposition and hatching rates, with fertility and fecundity decreasing in direct proportion to the concentration. A. indica nanoformulation has the potential to be a strategy for controlling malaria by reducing the longevity and reproductive capacity of the vector. Similarly (Baz et al., 2025) reported various plant extracts and found excellent results: Punica granatum (98.4 %) mortality, Citrus sinensis (92.0 %) mortality, Brassica oleracea (88.0 %) mortality, Oryza sativa (81.6 %) mortality, and Colocasia esculenta (53.6 %) mortality of Cx. Pipiens larvae after 24 h of application of the treatment.

Conclusion

This was concluded from the current study that botanical extracts, especially Neem and Citrus, possess potent larvicidal, ovicidal, and repellent properties against Anopheles stephensi. These effects were strongly concentration dependent but consistent across treatments, as indicated by non-significant interaction terms. The high effects of

Neem's exceptional efficacy are attributed to azadirachtin and related limonoids, which disrupt hormonal regulation, feeding, and metamorphosis in mosquitoes. Citrus oils, rich in limonene and other monoterpenes, act as repellents and ovicides through neurotoxic and membrane-disruptive mechanisms. Moringa and Nerium, while less potent, still demonstrated measurable biological activity, suggesting secondary roles in integrated vector control. Overall, these findings highlight that botanical extracts offer safe, eco-friendly, and cost-effective alternatives to synthetic insecticides. Their broad activity across mosquito life-stages supports their inclusion in Integrated Vector Management (IVM) programs, reducing reliance on chemical insecticides and mitigating resistance development.

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