ORIGINAL ARTICLE

Optimization and *in vitro* Characterization of Piperine-lemongrass Loaded Nanoemulsions Green Larvicide for *Aedes aegypti*

Pallavi Tiwari[1], Nisha Sharma[2], Anupriya Kapoor[1], Prakash Chandra Gupta[2]

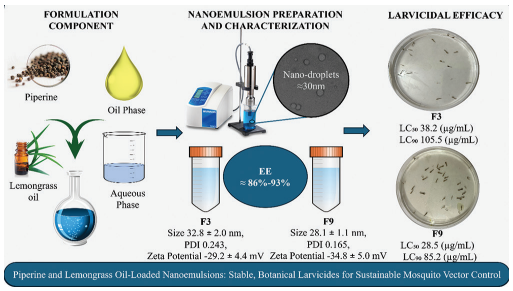
[1]Department of Phamaceutics, School of Pharmaceutical Sciences, Chhatrapati Shahu Ji Maharaj University, Kanpur, Uttar Pradesh, India, [2]Department of Pharmacognosy, School of Pharmaceutical Sciences, Chhatrapati Shahu Ji Maharaj University, Kanpur, Uttar Pradesh, India

Abstract

Aim: *Aedes aegypti* is a major vector for dengue and Zika. Rising resistance to synthetic pesticides has shifted focus toward botanical larvicides like piperine and lemongrass oil. However, these actives suffer from high hydrophobicity and environmental instability. This study aimed to develop stable, green nanoemulsions to enhance their delivery and larvicidal efficacy. **Material and Methods:** Active ingredients included piperine and lemongrass oil . The oil phase utilized olive oil, while Tween 80 and propylene glycol served as surfactant and co-surfactant. Distilled water formed the aqueous phase, with ethanol and methanol (S.D. Fine chemicals) used as analytical reagents. Triethanolamine and citric acid were employed for pH adjustment. Nine formulations (F1–F9) were designed using a 32 factorial model and prepared via low-energy emulsification followed by ultrasonication (650W, 20 kHz). Characterization was performed using Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), and UV-Vis spectrophotometry .**Results and Discussion:** Formulation F9 was identified as the lead system, containing 3.0% piperine and 3.0% lemongrass oil. It exhibited an optimal droplet size of 28.1±1.1 nm, a PDI of 0.165, and a zeta potential of −34.8±5.0 mV, indicating high colloidal stability. F9 demonstrated superior larvicidal potency with an LC50 of 28.5 µg/mL and 89% mortality after 48 hours. The nano-sized droplets improved penetration and contact with the larval cuticle, leading to physiological disruption and gut paralysis. **Conclusion:** The study successfully optimized a dual-active nanoemulsion that provides a 2- to 4-fold increase in efficacy over free botanical extracts. These green nanoemulsions represent a sustainable, biodegradable, and highly effective alternative for integrated mosquito management.

Key words: *Aedes aegypti*, Larvicidal activity, nanoemulsion, Lemon grass oil, piperine, biodegradable, mortality.

Graphical Abstract



INTRODUCTION

Mosquito-borne diseases are an important public health concern, particularly in tropical and subtropical areas where mosquitoes breed eagerly. *Aedes aegypti* is the key mosquito that spreads dengue, Zika, chikungunya, and yellow fever.[1,2] These mosquitoes live in urban areas and spread rapidly in water found in containers and old tires.[3] This makes it hard to control their population.[4,5] Outdated chemical larvicides are normally used to control breeding sites, but rising resistance and harm to the environment are

Address for correspondence:
Dr. Nisha Sharma, School of Pharmaceutical Sciences, Chhatrapati Shahu Ji Maharaj University, Kanpur - 2028024, Uttar Pradesh, India. Phone: +91-7007508359.
E-mail: nishasharma@csjmu.ac.in

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problems.[6,7] These chemicals can build up in the ecosystem, putting aquatic life at risk and affecting food chains, which shows the need for natural substitutes.[8,9]

These challenges remind us how important it is to find effective ways to protect our ecosystems while keeping diseases at bay. Botanical larvicides, made from plant compounds, are a great option because they break down easily and can target various processes at once.[10,11] For example, piperine from Piper plants interrupts larval neuroenzymes and metabolism at low concentrations (lethal concentration $[LC]_{50}$ 20–95 µg/mL).[12,13] Lemongrass oil (*Cymbopogon citratus*) is known for its active ingredient, citral, which can damage membranes and lead to suffocation at concentrations between 50 and 120 µg/mL and is relatively low in toxicity to mammals.[14,15] However, the effectiveness of lemongrass oil can be a bit limited because of its hydrophobic nature, volatility, and instability. This means that creating better formulations is important to make the most of its benefits.[16,17]

Nanoemulsions reduce the poor aqueous solubility, rapid degradation, and surface floating of piperine and lemongrass oil, by encapsulating actives in <200 nm surfactant-stabilized droplets.[18,19] This achieves stable colloidal dispersion, protects against oxidation/hydrolysis, enhances larval cuticle contact via reduced surface tension, and provides sustained release resulting in 2–4-fold LC_{50} reduction.[20,21]

This study focuses on optimizing and evaluating a combination of piperine and lemongrass oil nanoemulsions for enhanced effectiveness against *A. aegypti* larvae, to achieve environmentally friendly pest control.

MATERIALS AND METHODS

Chemicals

The key active ingredients include piperine and citral, lemongrass oil sourced from Yucca Enterprises (India). Formulation excipients Tween 80 and propylene glycol, both from Loba Chemie Pvt. Ltd. (India), along with olive oil as the oil phase and distilled water as the aqueous medium. Additional reagents for analysis and larvicidal activity work encompass ethanol and methanol from S.D. Fine chemicals, Mumbai.

Preparation of nanoemulsion

Low-energy emulsification method

Oil-in-water nanoemulsions were formulated with piperine (1.5–3% w/v), lemongrass oil (1.5–3% w/v), olive oil, distilled water, Tween 80 (7–12% v/v), propylene glycol (8% v/v), and stabilizers as per Table 1, detailing formulations F1-F9 with varying active ratios.[22] Piperine and lemongrass oil dissolved in the olive oil formed the oil phase; Tween 80 and propylene glycol mixed with distilled water to form the aqueous phase, at 25°C in screw-capped vials.[23] Oil phase added dropwise to aqueous under stirring formed pre-emulsion, afterwards ultrasonication (650W, 20 kHz, 5–10 min) through low-energy method yielding stable nanoemulsions (28–45 nm droplets).[24,25] The schematic [Figure 1] shows this low-energy emulsification process: Oil phase preparation on a hotplate, aqueous-surfactant equilibration at 25°C, slow addition to form a pre-emulsion under stirring, and final ultrasonication for the reduction of droplet size. This method minimizes the squalor of sensitive actives, ensuring colloidal stability for larvicidal use.[26]

Evaluation of nanoemulsion

Dynamic light scattering (DLS)

The droplet size, polydispersity index (PDI), and zeta potential of piperine-lemongrass oil nanoemulsions were measured using DLS instrument (Malvern Zetasizer Nano ZS).[27,28] To minimize multiple scattering effects and certify accurate readings, each sample was suitably diluted with distilled water.[29] All analyses were conducted at a controlled temperature of 25 ± 1°C in triplicate measurements, recording the mean hydrodynamic diameter (Z-average), PDI values (for distribution uniformity), and zeta potential (for colloidal stability calculation).[30,31]

Table 1: 3^2 Factorial design for piperine-lemongrass nanoemulsion compositions

Run	A: Piperine (%)	B: Lemongrass (%)	Oil phase (%)	Tween 80 (%)	PG (%)	Water (%)	Stabilizer (%)
F1	1.5	1.5	15	9	8	65	2
F2	1.5	2.25	17	10	8	62	3
F3	1.5	3.0	20	11	8	57	3
F4	2.25	1.5	17	11	8	62	2.5
F5	2.25	2.25	20	12	8	57	2.5
F6	2.25	3.0	22	13	8	53	2.5
F7	3.0	1.5	20	12	8	57	3
F8	3.0	2.25	22	13	8	53	3
F9	3.0	3.0	25	14	8	49	3

PG: Propylene glycol

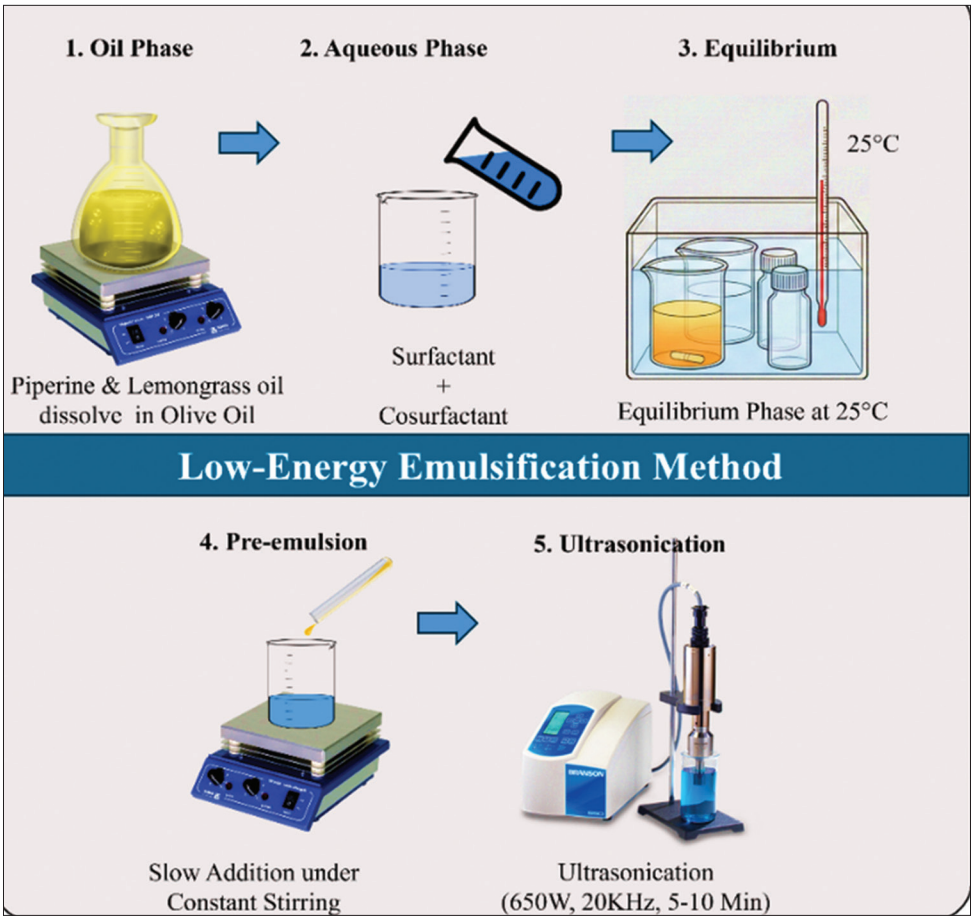

Figure 1: Schematic representation of the low-energy ultrasonication method for nanoemulsion preparation

Transmission electron microscopy (TEM)

To image nanoemulsion morphology using a high-resolution instrument, TEM operating at accelerating voltages of 160–220 kV was utilized. 1–2 drops of diluted nanoemulsion were placed on carbon-coated copper grids, allowed to dry, and negatively stained with 2% uranyl acetate or phosphotungstic acid following well-known protocols.[32,33] Imaging was achieved at magnifications ranging from ×50,000 to ×200,000, depending on droplet size, with scale bars included in micrographs; examinations at room temperature well-kept-up the native vesicular structure.[34,35]

Drug content estimation

Drug content and entrapment efficiency for piperine and lemongrass oil were measured by extracting a pre-weighed quantity of nanoemulsion in a suitable solvent, like methanol, to attain complete dissolution of the active ingredients.[36-38] The extract was centrifuged to separate unentrapped drug, and the supernatant analyzed spectrophotometrically at λ_{max} (piperine 343 nm, lemongrass oil 238 nm) using a Ultraviolet (UV)-Vis spectrophotometer (Shimadzu, PharmaSpec-1700).[39,40] Standard calibration curves (prepared with blanks) enabled precise quantification; all determinations were performed in triplicate to verify reproducibility and precision.[41,42]

Larvicidal activity

The larvicidal potential of dual delivery of piperine-lemongrass formulations (F3 and F9) against third-instar larvae of *A. aegypti* was measured according to World Health Organization (WHO) standard guidelines.[43,44] Twenty healthy, synchronized third-instar larvae were shifted to 100 mL beakers holding 99 mL distilled water plus 1 mL test nanoemulsion (three replicates per concentration).[45,46] Environmental parameters upheld: 27 ± 2°C temperature, 65–70% relative humidity, 12:12 h light: dark cycle.[12] Post-exposure at 24 and 48 h, larvae screening no response to gentle probing were deemed dead; moribund individuals that weakened to reach the water surface were calculated as mortality.[47] Percentage mortality calculated by Abbott's formula for controls (5–20%); LC_{50} and LC_{90} resultant from probit analysis of dose-response data.[48,49]

RESULTS AND DISCUSSION

Preparation of nanoemulsion

Nine piperine-lemongrass oil nanoemulsions (F1–F9) were successfully developed as per the 3^2 factorial design

in Table 1, using piperine (1.5–3%) and lemongrass oil (1.5–3%) with olive oil phase (15–25%). Aqueous phase made up of Tween 80 (9–14%) and propylene glycol (8%); pre-emulsions formed by dropwise addition under stirring, polished by ultrasonication (650 W, 20 kHz, 5–10 min). All produced translucent, stable nanoemulsions with pH 5.0–6.8 (adjusted with triethanolamine/citric acid) and q.s. to 100 mL distilled water.

DLS

The optimized piperine-lemongrass nanoemulsion F9 [Figure 2] (28.1 ± 1.1 nm, PDI 0.165, −34.8 ± 5.0 mV) established greater colloidal stability with nano-sized droplets, greater monodispersity (PDI <0.2), and strong electrostatic repulsion (zeta −34 mV), making it the lead formulation from the 3^2 factorial design [Table 2].

F3 (32.8 ± 2.0 nm, PDI 0.243, −29.2 ± 4.4 mV) demonstrated acceptable yet below-average stability, following the pattern of reduced droplet size as Tween 80 concentration increased, and was outperformed by F9's enhancements of approximately 14–20% across all formulations. The low polydispersity suggests uniform distributions that reduce the likelihood of Ostwald ripening, while the zeta potentials provide repulsion to prevent coalescence.

The ideal surfactant concentration was 14% Tween 80 for formulation F9 (comprising 3.0% piperine and 3.0% lemongrass with a 25% oil phase), compared to a less effective 11% Tween 80 for formulation F3 (which included 1.5% piperine and 3.0% lemongrass). The droplet size significantly reduced from formulation F1, which had a size of 41.2 nm at 9% Tween 80.

F6 showed moderate stability (46.9 ± 3.1 nm, PDI 0.271, −22.7 ± 3.8 mV) due to high oil content indorsing coalescence. F9 with 14% w/v Tween 80 was found to be optimal for stable piperine-lemongrass nanoemulsions appropriate for larvicidal applications.

TEM

The TEM of optimized formulation of the piperine-lemongrass nanoemulsion revealed predominantly spherical particles with smooth surfaces and a consistent size distribution. Among the optimized formulations, F9 exhibited the most favorable morphological characteristics, with particle sizes ranging from approximately 28 to 30 nm, as illustrated in Figure 3. The TEM observation confirms the DLS data, supporting the uniformity of the nano-sized particles and demonstrating an effective method for controlling *Aedes* vectors.

Drug content estimation

Drug content measurement for both piperine and lemongrass oil in formulations F3 and F9 was carried out using UV-Vis spectrophotometry at their own maximum absorption wavelengths. The calibration curves showed excellent linearity ($R^2 > 0.999$).

Both formulations showed imposing dual-active recovery rates, ranging from F3: 86.5–88.0% to F9: 91.7–93.0%. This shows effective entrapment of the piperine-lemongrass combinations, with processing losses remaining within the characteristic range for nanoemulsion systems.

The recovery rates (F3: 86.5–88.0%–F9: 91.7–93.0%) across both actives in F3 and F9 show efficient emulsification for dual-active piperine-lemongrass nanoemulsions, with minor losses attributable to sonication processing, oil division, or UV absorbance matrix effects during examination. These results confirm the factorial design formulations attained target loadings within pharmacologically satisfactory limits for phytoconstituent nano-delivery systems.

Larvicidal activity

Formulations F9 and F3 showed dose-dependent larvicidal activity against third-instar *A. aegypti* larvae, as shown in

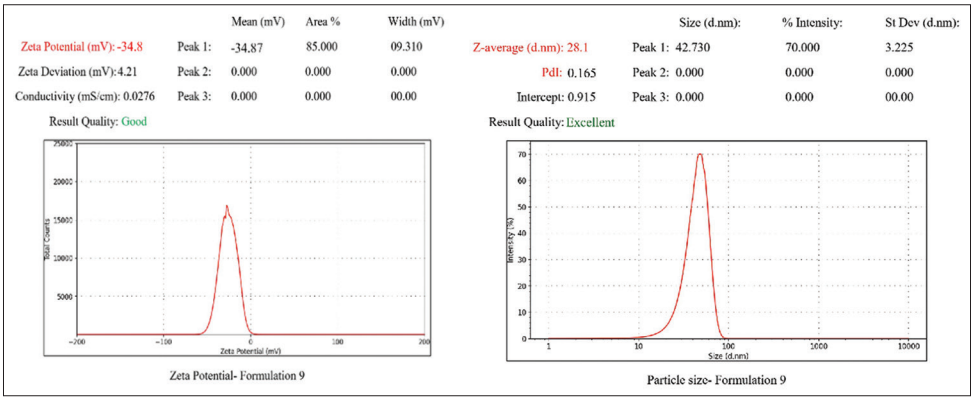

Figure 2: Dynamic light scattering representation of the piperine and lemongrass oil-loaded nanoemulsion (F9)

Table 3. F9 (piperine 3.0% + lemongrass 3.0%) established satisfactory strength over F3 (piperine 1.5% + lemongrass 3%), attaining 89% mortality at 90 µg/mL (48 h) compared to F3 requiring 120 µg/mL for equal effect (85% mortality). Both formulations showed advanced mortality increases from 24 to 48 h across established concentrations (30–120 µg/mL).

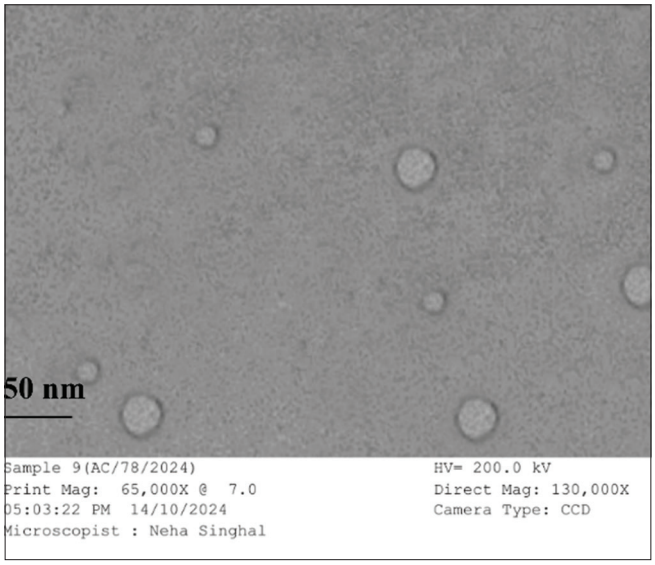

Figure 3: Transmission electron microscopy analysis of formulation F9

Table 2: DLS characterization of piperine-lemongrass nanoemulsions (*n*=3)

Formulation code	Particle size (Z-average, nm)	PDI	Zeta potential (mV)
F1	41.2±2.0	0.252	−24.8±3.9
F2	38.1±2.0	0.225	−27.5±4.2
F3	32.8±2.0	0.243	−29.2±4.4
F4	44.6±2.8	0.241	−24.1±4.3
F5	38.5±2.3	0.226	−27.0±4.1
F6	46.9±3.1	0.271	−22.7±3.8
F7	33.9±2.1	0.229	−28.5±4.5
F8	37.4±2.6	0.251	−26.3±4.2
F9	28.1±1.1	0.165	−34.8±5.0

DLS: Dynamic light scattering, PDI: Polydispersity index

Observed behavioral changes reduced mobility, increased agitation, and distress signals [Table 3 and Figure 4] (F9/F3-treated larvae showing dead third-instars with disrupted morphology) indicate enhanced physiological disruption through greater surface contact and sustained piperine-lemongrass release from F9 nanoemulsion, overpowering free phytoconstituents aqueous solubility limitations.

LC

F9 showed lower LC_{50} (28.5 µg/mL) and lower LC_{90} (85.2 µg/mL) compared to F3 (LC_{50}: 38.2 µg/mL; LC_{90}: 105.5 µg/mL), representing superior effectiveness across the whole dose range despite higher active loading.

F9 superiority across characterization [Tables 2, 4, and 5] from optimal high-surfactant design (14% Tween 80, 25% oil phase) with higher piperine-lemongrass concentrations (6.0% total actives), attractive bioavailability, and more effectively than F3 (11% Tween 80, 4.5% total actives).

The low LC_{50} values (<50 µg/mL) for both formulations confirm nanoemulsion encapsulation improves piperine-lemongrass larvicidal performance over free equivalents reported in literature (20–95 µg/mL piperine, 50–120 µg/mL lemongrass oil), with F9 achieving 3-fold development and validating the very active loading nano-delivery method for practical vector control applications. Table 6 shows the LC values for piperine-lemongrass nanoemulsions F9 and F3 against third-instar *A. aegypti* larvae.

Table 3: Larvicidal activity of the formulations

Formulation	Dose (µg/mL)	Mortality 24 h (%)±SD	Mortality 48 h (%)±SD	Observations
F9 (3.0% piperine+3.0% lemongrass)	30	25±1.2	52±1.1	Reduced mobility
	60	42±1.0	72±0.9	Agitation, gut paralysis
	90	58±0.8	89±0.7	Distress, surfacing failure
	120	68±0.9	96±0.5	Total immobility
F3 (1.5% piperine+3.0% lemongrass)	30	18±1.1	38±1.0	Reduced mobility
	60	32±1.1	58±0.9	Moderate agitation
	90	45±1.0	74±0.8	Distress signs
	120	55±1.0	85±0.7	Immobility

SD: Standard deviation

Table 4: Drug content of F3 (piperine 1.5%+lemongrass 3% w/v)

Active ingredient	Initial conc. (% w/v) taken	Measured absorbance (nm)	Calculated conc. (% w/v)	Obtained % drug content
Piperine	1.5	0.335	1.30	86.6
Lemongrass oil	3.0	0.409	2.64	88.0

Table 5: Drug content of F9 (piperine 3.0%+lemongrass 3.0% w/v)

Active ingredient	Expected conc. (% w/v)	Measured absorbance (nm)	Calculated conc. (% w/v)	Obtained % drug content
Piperine	3.0	0.359	2.75	91.7
Lemongrass oil	3.0	0.328	2.79	93.0

Table 6: LC_{50} and LC_{90} of the formulations F3 and F9

Formulation code	LC_{50} (µg/mL)	LC_{90} (µg/mL)
F3 (piperine 1.5+lemongrass 3.0)	38.2±2.4	105.5±4.1
F9 (piperine 3.0+lemongrass 3.0)	28.5±2.1	85.2±3.8

LC: Lethal concentration

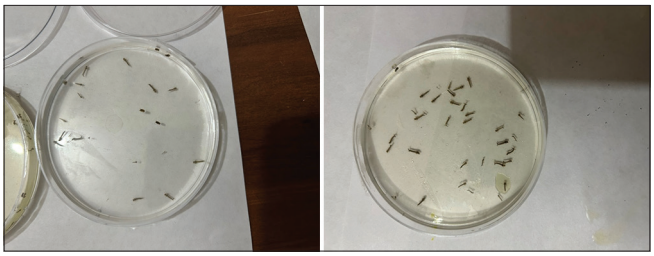

Figure 4: Larvicidal activity of optimized F3 and F9 nanoemulsions against mosquito larvae, respectively

CONCLUSION

The development of piperine-lemongrass oil nanoemulsions through 3^2 factorial design optimization produced F9 as the dominating formulation (3.0% piperine + 3.0% lemongrass oil), while F3 (1.5% piperine + 3.0% lemongrass oil) served as an effective proportional benchmark.

F9 demonstrated optimum physicochemical characteristics: smallest droplet size (28.1 ± 1.1 nm), lowest PDI (0.165), highest zeta potential (−34.8 ± 5.0 mV), and outstanding entrapment (91.7–93.0%). F3 exhibited good performance (32.8 ± 2.0 nm, PDI 0.243, −29.2 ± 4.4 mV, 86.5–88.0% entrapment).

In WHO-standardized larvicidal assays against third-instar *A. aegypti*, F9 brought superior dose-dependent mortality (30–120 µg/mL), achieving 89% mortality at 90 µg/mL (48 h) related to F3, 85% mortality at 120 µg/mL, with behavioral disruptions (agitation → paralysis → suffocation) indicative of synergistic neuro-metabolic interference.

F9 showed evidently enhanced potency (LC_{50} 28.5 µg/mL, LC_{90} 85.2 µg/mL) 1.3-fold higher to F3 (LC_{50} 38.2 µg/mL, LC_{90} 105.5 µg/mL) justified by higher active loading (6.0% vs. F3 4.5%) and optimized Tween 80 (14% vs. F3 11%) oil (25% vs. F3 20%) interactions indorsing superior bioavailability.

These LC_{50} values (F9: 28.5 µg/mL; F3: 38.2 µg/mL) exceed the literature benchmarks for free piperine (20–95 µg/mL) and lemongrass oil (50–120 µg/mL) by 2–4-fold, confirming that nanoencapsulation transforms phytoconstituents into high-efficacy, stable larvicides. Both formulations confirm the nano-delivery method, but F9 characterizes the transformative platform for sustainable vector control with <1 ppm field dosing capability.

These findings highlight herbal nanoemulsions as a promising botanical larvicide platform that not only improves efficacy but also aligns with environmental compatibility, particularly compared to synthetic larvicides, which frequently lead to resistance and exhibit non-target toxicity. As natural and decomposable phytoconstituents, piperine and lemongrass oil exemplify the principles of green nanotechnology by contributing to reduced ecological impact and greater safety for non-target organisms. This positions piperine-lemongrass nanoemulsions as superior contenders for the next generation of sustainable, resistance-mitigating combined vector management programs.

AUTHOR CONTRIBUTIONS

PT: Substantial contributions to conception and design, drafting the article, NS: Final approval of the version to be published, AK: Revising it critically for important intellectual content. PCG: Formal analysis.

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