LARVICIDAL ACTIVITY OF DIFFERENT MOLECULAR WEIGHTS CHITOSAN-PLANT EXTRACT COMPLEX AGAINST *ANOPHELES STEPHENSI*

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Abstract

Molecular weights of chitosan are important to their physical and biological features. The present study aims to compare the larvicidal efficacy of nanoparticles prepared with low (L-CS) and medium molecular weight (M-CS) chitosan, encapsulated with methanolic aerial part extract (MAE) of Artemisia absinthium (chitosan-plant extract complex) against Anopheles stephensi. They were characterised in terms of entrapment efficiency, drug release percentage, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and zeta potential. Out of various concentrations of L-CS- and M-CS-based nanoparticles, MAEL-CSNPs and MAEM-CSNPs, respectively, 10 µg/mL MAEL-CSNPs exhibited higher entrapment efficiency (64.10%). In vitro drug release study showed a controlled and sustained crude drug release from MAEL-CSNPs (86.60%) within three hours, with the best R^2 value observed in the Higuchi model (0.97). Spectroscopically characterised MAEL-CSNPs with the lowest LC_{50} value (2.99 µg/mL) were subjected to toxicity analysis using zebrafish embryos, and the result indicates that, for the encapsulated drug, below 100% mortality at 10 µg/mL concentration and it may act as a potent larvicide without harming non-targeted organisms. Thus, the results showed the capability of MAEL-CSNPs to act as a potent, eco-friendly larvicide against An. stephensi larvae.

Keywords: *larvicidal activity, methanolic aerial part extract, Artemisia absinthium, low and medium molecular weight chitosan nanoparticles, Anopheles stephensi*

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1. Introduction

The health sector and entomologists have been facing various challenges to protect humankind from harmful mosquitoes. Mosquitoes are primarily responsible for the spread of several intimidating vector-borne diseases, namely dengue, lymphatic filariasis, chikungunya, malaria, Japanese encephalitis and yellow fever [1]. Considering the escalating populations of vector mosquitoes and cases of vector-borne diseases, immediate control strategies are needed to improve the usefulness of control measures [2]. Malaria is one of the severe infectious diseases that leads to the suffering of about half a billion people each year worldwide [3]. This disease is preventable and curable, and many anti-malarial studies have been conducted with herbal-based products. However, more notice is still needed due to the rapid mutations in malaria receptor structures when competing with each drug [3]. Apart from plant-derived compounds, green-synthesised nanoparticles (NPs) from different plant parts have been considered an innovative approach for controlling mosquito population in an eco-friendly manner [4]. Polymeric nanoparticles offer a new advancement in drug discovery and can be made from artificial or natural polymers. Chitosan is a propitious biopolymer for drug delivery systems. Because of its desirable properties, chitosan is extensively used in the biomedical and pharmaceutical sectors. Chitosan is viewed as the most significant polysaccharide for various drug delivery purposes due to its cationic character and primary amino groups, which are responsible for its different properties such as mucoadhesion, controlled drug release, transfection, *in situ* gelation, efflux pump inhibitory properties and permeation enhancement [5]. Based on molecular weight, chitosan can be classified into low molecular weight (< 150 kDa), medium molecular weight (150 - 700 kDa), and high molecular weight (> 700 kDa). The physical, chemical and biological features of chitosan mainly depend on the degree of deacetylation and molecular weight [6]. High molecular weight chitosans show poor solubility and high viscosity at neutral pH. In contrast, low molecular weight chitosans show more solubility and less viscosity, with easy membrane penetration at neutral pH. Because of these properties, various studies have been carried out using this low molecular weight chitosan as a biological stimulant, antibacterial material, antifungal agent or antioxidant [7–11].

Artemisia absinthium L. (Asteraceae) is native to temperate regions of North America, South Africa, Europe and Asia. The plant was reported in folkloric medicine for its repellent property against moths, lice and bed bugs [12]. It is also a rich source of terpenes, antioxidants, phenolics, flavonoids and various biologically active compounds [13]. *A. absinthium* contains several phytochemical compounds, namely, lactones, terpenoids (e.g., *trans*-thujone, γ-terpinene, 1,4-terpeniol, myrcene, bornyl acetate, cadinene, camphene, *trans*-sabinyl acetate, guaiazulene, chamazulene, camphor, and linalool), essential oils, organic acids, resins, tannins, and phenols [14]. In addition, it was used as an anthelmintic and insect repellent [15]. Larvicidal activity of leaf extract (methanolic and ethanolic) of *A. absinthium* was used to determine toxicity against *Aedes aegypti* [16]. Larvicidal, pupicidal, and adulticidal effects of acetone, chloroform and methanolic leaf and stem extracts of *A. absinthium* were evaluated against *Ae. aegypti* [17]. Efficacy of the leaf extract of *A. annua* was studied by Preethi *et al*. [18] against *An. stephensi*. The EC_{50} value of the leaf extract of *A. cina* was assessed against *Cx. pipens* [19]. Based on the previous literature, there is no work on the larvicidal efficacy of low and medium molecular weight chitosan nanoparticles encapsulated with methanolic aerial part extract (chitosan-plant extract complex) of *A. absinthium* against *Anopheles stephensi*. Therefore, the present investigation was undertaken.

2. Materials and Methods

2.1. Materials

Methanol (99.5%) extra pure was purchased from Loba Chemie Pvt. Ltd., Mumbai, India; benzene (99.5%) and acetone (99.5%) of analytical grade were procured from Spectrum Reagents and Chemicals Pvt. Ltd., Edayar Cochin, India. Low (< 150 kDa) (L-CS) and medium (150 - 700 kDa) (M-CS) molecular weight extra pure chitosan, sodium tripolyphosphate (TPP), glacial acetic acid, dialysis membrane bag, phosphate buffer, temephos, and microtiter plates were procured from Sisco Research Laboratories (Mumbai, India).

2.2. Collection, Identification and Preparation of the Plant

A. absinthium was collected from Coimbatore district, Tamil Nadu, India. The plant was identified and authenticated in the Botanical Survey of India, Coimbatore district, Tamil Nadu (BSI/SRC/5/23/2020/Tech). The aerial part of the plant was shade-dried in hygienic conditions for 4 - 5 days. Thereafter, the aerial part was powdered in an electrical grinder machine and carefully stored for further processing.

2.3. Extraction Process

A simple cold maceration process using three solvents, benzene, acetone and methanol, was performed to extract the aerial part of the plant. Ten grams of powder were mixed with 100 mL of solvents separately and were kept for 24 hours in an orbital shaker. Then, the extracts were filtered through Whatman No. 1 filter paper. Finally, the solvent was evaporated using a rotary evaporator to obtain the concentrated mass. Then the extracts were stored in the refrigerator for further use.

2.4. Mosquito Larvicidal Bioassay

An. stephensi larvae were collected from the National Centre for Disease Control (NCDC) field station at Mettupalayam, Coimbatore district, Tamil Nadu. The early fourth instar larvae of *An. stephensi* were taken for the larvicidal bioassay, which was carried out according to the WHO standard with slight modifications (1996) [20]. For the experiments, larvae were maintained in trays containing tap water and regularly fed with a diet of brewer's yeast and dog biscuits in the ratio of 3:1. All the experiments were carried out at 27 ± 2°C and 75 - 85% relative humidity under 14:10 hours of light:dark cycles. Larvae were introduced to all the solvent extracts at three different concentrations of 0.75, 1.50, and 3.00 mg/L along with control Temephos (1 mg/L) and negative control (methanol, acetone, and benzene). The larval toxicity assays were replicated four times, and the mortality of larvae was recorded every 24 h.

2.5. Synthesis of Methanolic Aerial Part Extract Encapsulated Chitosan Nanoparticles

L-CS- and M-CS-based nanoparticles containing methanolic aerial part extract (MAE), denoted as MAEL-CSNPs and MAEM-CSNPs, respectively, were prepared using the ionic gelation method [21]. Briefly, 0.5% (w/v) chitosan was dissolved in 1% glacial acetic acid and stirred until the solution became transparent. Then, the pH was adjusted to 5.0 at room temperature. MAE at five different concentrations (10, 20, 30, 40, and 50 µg/mL) was separately added to L-CSNPs and M-CSNPs to prepare the chitosan-plant extract complex. Then, 0.1% of the sodium tripolyphosphate solution (TPP) was added dropwise to the mixture and stirred until an opalescence was noted. The mixtures were centrifuged at 10000 rpm for 15 min and washed twice with deionised water. After centrifugation,

the supernatants were discarded, and the pellets containing MAEL-CSNPs and MAEM-CSNPs were stored in the refrigerator at 4°C for further use.

2.6. Determination of Entrapment Efficiency

The amount of MAE encapsulated within the L-CSNPs and M-CSNPs was determined by an indirect method, by calculating the amount of unencapsulated drug. After adding the TPP, the mixtures were centrifuged at 10000 rpm for 15 min, and the clear supernatants containing the free unencapsulated drugs were collected, diluted with distilled water and measured spectrophotometrically at 273 nm [22]. The drug (MAE) entrapment or encapsulation efficiency (EE, %) in L-CSNPs and M-CSNPs was determined using the following equation:

$$\mathrm{EE} = \frac{(\mathrm{MAE\ added} - \mathrm{MAE\ in\ supernantant}) \cdot 100\%}{\mathrm{MAE\ added}} \quad (1)$$

2.7. Determination of *in vitro* Drug Release Percentage

The percentage of MAE released from MAEL-CSNPs and MAEM-CSNPs was estimated using the dialysis tube analysis technique (12,000-14,000 g/mol molecular weight). In brief, the dialysis membrane was washed with lukewarm double-distilled water (70°C) for 1 h and rinsed thoroughly (thrice) to remove glycerine. MAEL-CSNPs and MAEM-CSNPs with higher entrapment efficiencies (86.6 and 80.0%, respectively) were immersed separately in the dialysis bags containing 50 mL of phosphate buffer (pH 7.4) at room temperature with stirring at 1000 rpm. 3 mL of the solution was withdrawn every half an hour and replaced with an equivalent volume of fresh solution. This process was repeated for up to 5 h. The withdrawn samples were analysed using UV/visible spectroscopy at 265 nm, and the amount of MAE release patterns from MAEL-CSNPs and MAEM-CSNPs was determined [23]. The drug release percentage was determined using the following formula:

$$\text{Drug release} = \frac{C_t}{C_0} \cdot 100\% \quad (2)$$

where:

C_t – is the drug concentration calculated from the absorbance at 265 nm at time t,

C_0 – is the initial drug concentration.

2.8. *In vitro* Drug Release Kinetics

Different release kinetic models, such as zero order, first order, Higuchi and Korsmeyer-Peppas, have been used to fit the cumulative *in vitro* drug release data and to explain the drug release kinetics [24]. The best model of release pattern was evaluated according to the highest determination coefficient (R^2) value [25].

2.9. Characterisation of Methanolic Extract Encapsulated Chitosan

The synthesised methanolic extract encapsulated chitosan nanoparticles, MAEL-CSNPs and MAEM-CSNPs, were subjected to Fourier transform infrared spectroscopy (ATRS-FTIR Shimadzu AIM 9000) for identifying functional groups present in these materials. The size and morphology of chitosan nanoparticles were investigated through scanning electron microscopy (Quanta 400 ESEM). The particle size of the nanoparticles was also assessed using an X-ray diffractometer (Shimadzu LabX-XRD 1600) based on the Scherrer equation. The physical stability of nanoparticles was determined using a zeta potential analyser (Malvern Panalytical, Chennai, India).

2.10. Mosquito Larvicidal Activity of Encapsulated Chitosan Nanoparticles

The larval toxicity assays were carried out using early fourth-instar larvae of *An. stephensi.* The larvae of *An. stephensi* were purchased from the Centre for Research in Medical Entomology, Madurai, Tamil Nadu, India. Larvae were placed in enamel trays containing dechlorinated water and maintained under laboratory conditions. The research experiment was conducted according to standard WHO procedure (WHO, 1996). The larvicidal assays with treatments such as MAEL-CSNPs and MAEM-CSNPs were carried out according to the standard guidelines. A total of 10 larvae were introduced into a glass beaker with tap water (250 mL) with the required concentration of the sample (100 µg/mL). Three replicates were done, and a negative control (methanol and water) was also maintained. The larval mortality percentage was calculated after 24 h of exposure. The control mortalities were also corrected using Abbott's formula [26].

2.11. Zebrafish Embryo Toxicity Assay

The sample exhibiting the highest larvicidal activity (MAEL-CSNPs) and the control (MAE) were subjected to toxicity analysis using the zebrafish model. The zebrafish embryos were collected from laboratory-adapted zebrafish (*Danio rerio*) (1:2). After 12 hours of fertilisation, the embryos were taken and rinsed with clear water for the toxicity assessment, and unfertilised eggs were discarded. The zebrafish embryos were kept in a 24-well plate and treated with 10 µg of MAEL-CSNPs for incubation. The mortality, embryo hatchability and malformations of the treated and control groups were observed every 0, 6, 12, 24 and 48 hours [27].

2.12. Statistical analysis

The average larval mortality data were subjected to Probit analysis for calculating LC_{50}, LC_{90} and other statistics at 95% fiducial limits, and the upper confidence limit, lower confidence limit and chi-square values were calculated using the SPSS 11.5 (Statistical Package of Social Sciences) software. Results with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Larvicidal Activity of *A. absinthium* Extracts Against *An. stephensi* Larvae

Among three extracts of aerial plant parts (obtained using benzene, acetone and methanol) that were tested at three different concentrations (0.75, 1.50 and 3.00 mg/l), the mortality rates of *An. stephensi* within 24 hours varied with the dose. Among the tested plant extracts, the methanolic extract showed the highest mortality rate, with 3 mg/L resulting in 100% mortality compared to the other two concentrations (Table 1). The 95% confidence limits of LC_{50}, LC_{90}, chi-square value and degrees of freedom were also calculated based on the mortality rates of larvae. The lowest LC_{50} was exhibited by methanolic extract (1.170 mg/L) compared to benzene (2.410 mg/L) and acetone (1.714 mg/L). Similarly, the lowest LC_{90} value was shown by the methanolic extract (1.819 mg/L) compared to other extracts (2.739 mg/L and 4.322 mg/L, respectively). The degrees of freedom (DF) and the chi-square analysis of mortality with the use of methanolic extract were 2.0 and 1.261, respectively.

Table 1. Larvicidal activity of *A. absinthium* solvent-based plant extracts against *An. stephensi* larvae after 24 hours.

Plant extract	C (mg/L)	Mortality [%]	LC_{50} [mg/L]	LCL [mg/L]	UCL [mg/L]	LC_{90} [mg/L]	$\chi 2$	DF
Methanol	0.75	42.75	1.170	1.035	1.317	1.819	1.261	2.0
	1.50	80.00						
	3.00	100.00						
Acetone	0.75	36.75	1.714	1.470	1.985	2.739	2.046	1.0
	1.50	65.00						
	3.00	85.50						
Benzene	0.75	23.00	2.410	2.114	2.836	4.322	1.636	1.0
	1.50	44.00						
	3.00	68.50						
Positive Control		4.33	-	-	-	-	-	-

Note. Negative control nil mortality; C, extract concentration; LC_{50}, lethal concentration 50, referring to the concentration of a substance that is lethal to 50% of the population; LC_{90}, lethal concentration 90, referring to the concentration of a substance that is lethal to 90% of the population; LCL, lower confidence limit; UCL, upper confidence limit; χ^2, chi-square value; DF, degrees of freedom.

3.2. Entrapment Efficiency

Drug encapsulation or entrapment efficiency of L-CSNPs and M-CSNPs with methanolic aerial plant extract (MAE) is presented in Figure 1. Among various concentrations, low molecular weight chitosan-based nanoparticles encapsulated methanolic extract (MAEL-CSNPs) at 10 µg/mL showed higher entrapment efficiency (64.10%) compared to medium molecular weight chitosan-based nanoparticles encapsulated methanolic extract (59.34%).

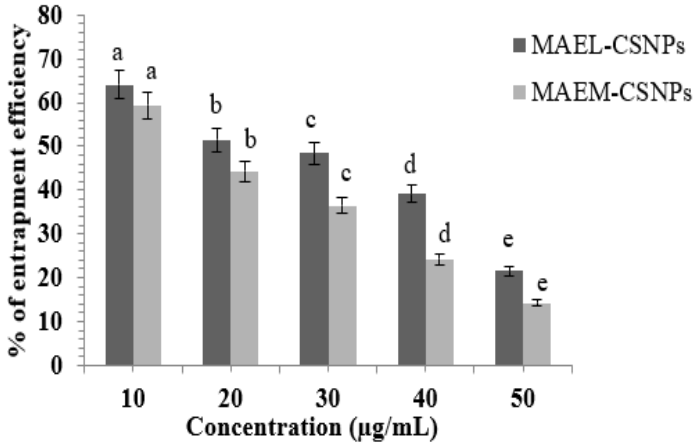


Figure 1. Entrapment efficiency of different concentrations of MAE encapsulated in CSNPs. Values are expressed as mean ± SD of three replicates. Statistically significant values ($p < 0.05$) are different according to Duncan's Multiple Range Test, where a > b > c > d > e.

3.3. *In vitro* Drug Release Kinetics

The MAEL-CSNPs and MAEM-CSNPs samples exhibiting the highest (64.10 and 52.34%, respectively) drug entrapment efficiency percentage values were subjected to *in vitro* drug release kinetics using four mathematical models: zero order, first order, the Higuchi model and the Korsmeyer-Peppas model (Figure 2). The determination coefficients of kinetic models are presented in Table 2. The Higuchi model with R^2 values of 0.978 and 0.893 for MAEL-CSNPs and MAEM-CSNPs was the best-fit model.

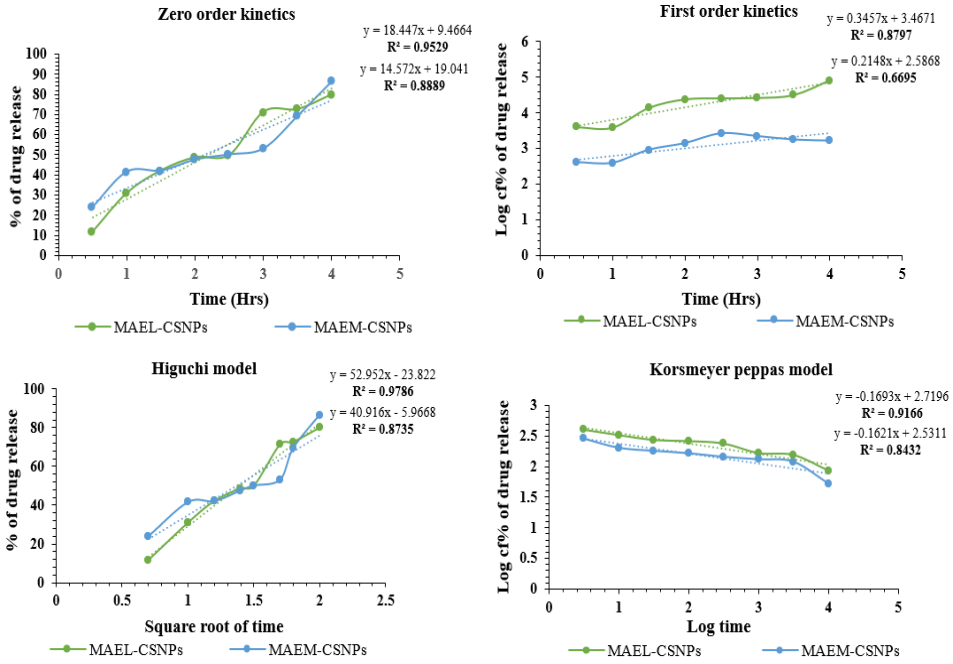


Figure 2. *In vitro* drug release kinetics models.

Table 2. Determination coefficients and kinetic constants of curves fitted using different kinetics models for *A. absinthium* encapsulated nanochitosan.

	MAEL-CSNPs			MAEM-CSNPs		
Kinetic model	**R^2**	**slope**	**intercept**	**R^2**	**slope**	**intercept**
zero-order	0.952	18.447	9.466	0.888	14.572	19.041
first-order	0.879	0.359	3.449	0.669	0.214	2.586
Higuchi	0.978	52.952	–23.822	0.893	–0.162	–2.531
Korsmeyer-Peppas	0.916	–0.169	2.719	0.843	40.196	–5.966

3.4. FTIR Analysis

The spectra of both MAEL-CSNPs and MAEM-CSNPs were quite similar except for the differences in band intensity (Figure 3). The bands at 1533, 1378, 1061, and 1023 cm^{-1} in the FTIR spectrum of MAEL-CSNPs (Figure 3a) are less intense when compared to the FTIR spectrum of MAEM-CSNPs (Figure 3b).

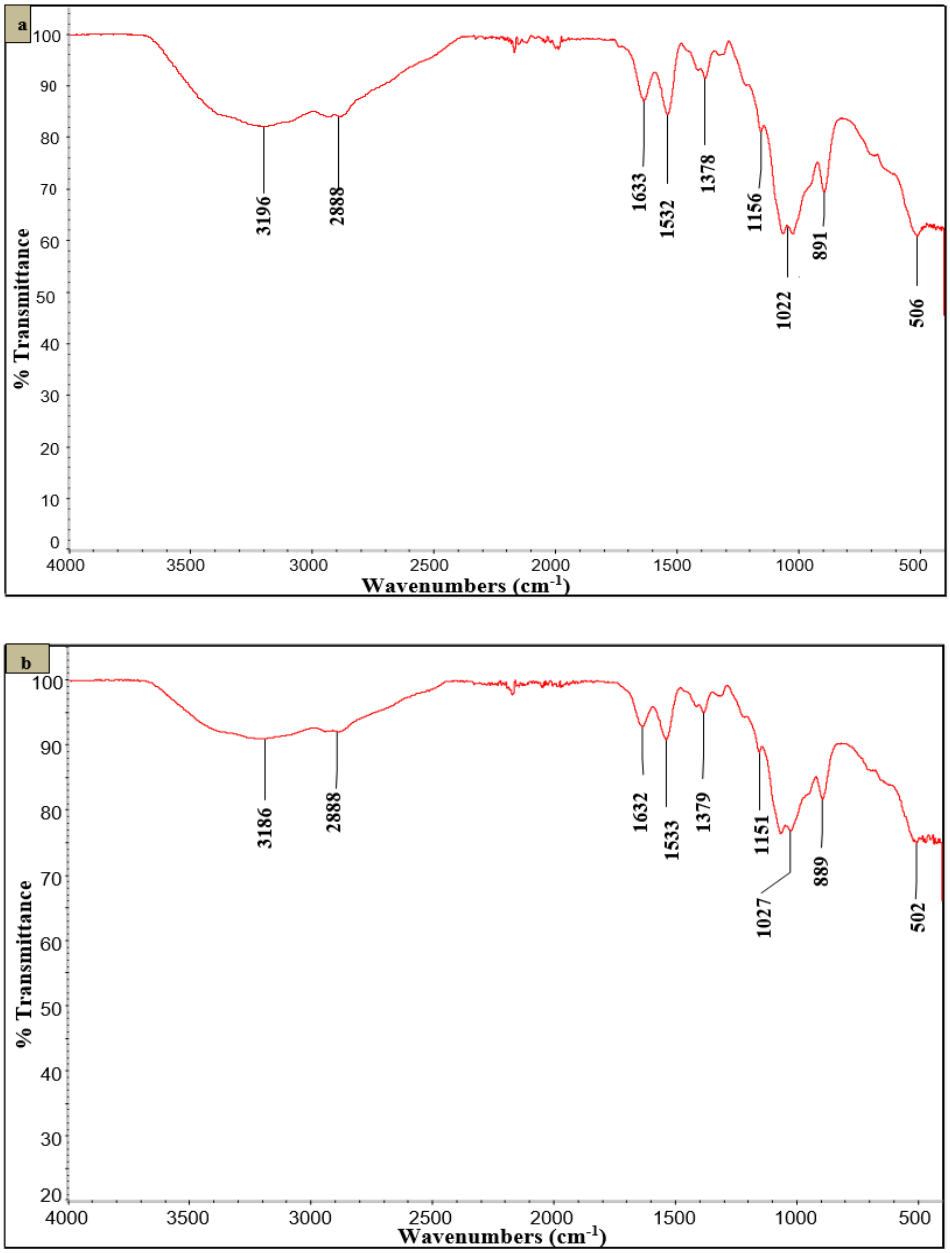


Figure 3. FTIR spectra of (a) MAEL-CSNPs and (b) MAEM-CSNPs.

3.5. XRD Analysis

Figure 4 shows XRD diffractograms of MAEL-CSNPs and MAEM-CSNPs. The comparison of results indicates differences in position and intensity of peaks. The characteristic peaks at 18.44°, 19.39°, 20.51°, and 22.50° of 809, 816, 783, and 844 intensity, respectively, in MAEL-CSNPs diffractogram (Figure 4a) are less intense and slightly shifted in the diffractogram of MAEM-CSNPs (Figure 4b), i.e., to 18.49°, 19.48°, 22.5°, and 23.5° with 940, 946, 970, and 984 intensity, respectively.

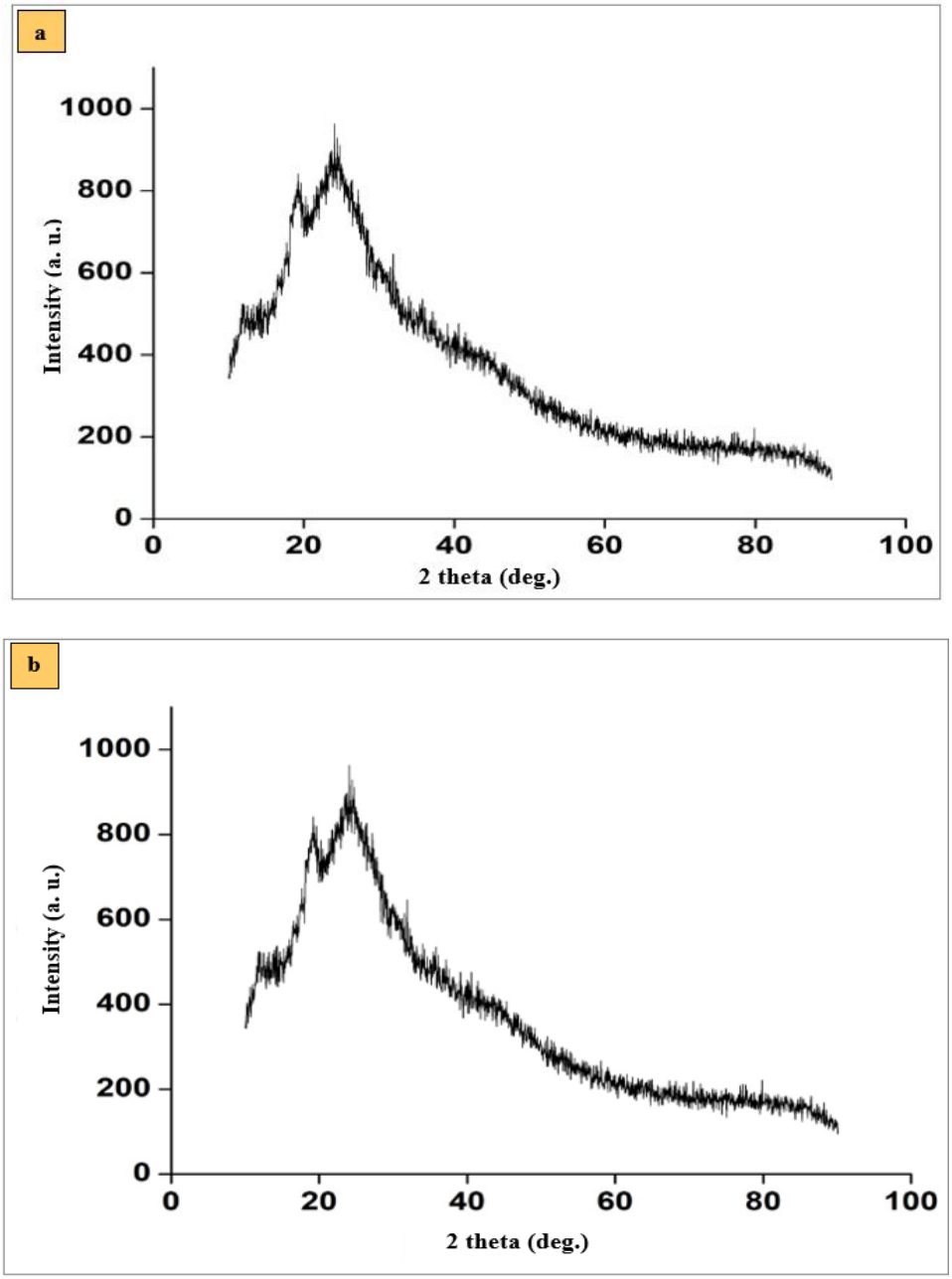
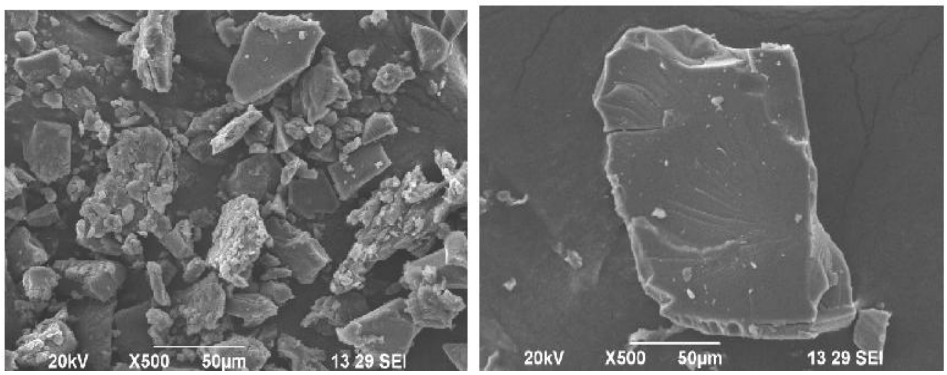


Figure 4. XRD diffractograms of (a) MAEL-CSNPs and (b) MAEM-CSNPs.

3.6. SEM Analysis

Figure 5 represents the SEM images of MAEL-CSNPs and MAEM-CSNPs at a 50 μm scale bar. Both MAEL-CSNPs and MAEM-CSNPs presented a homogenous topographic surface, but MAEL-CSNPs showed smaller nanoparticles than MAEM-CSNPs.



(a) **(b)**

Figure 5. SEM micrographs of (a) MAEL-CSNPs and (b) MAEM-CSNPs.

3.7. Zeta Potential

In zeta potential analysis, MAEM-CSNPs showed a higher charge, i.e., +43.2 mV, than MAEL-CSNPs, i.e., +30.8 mV (Figure 6).

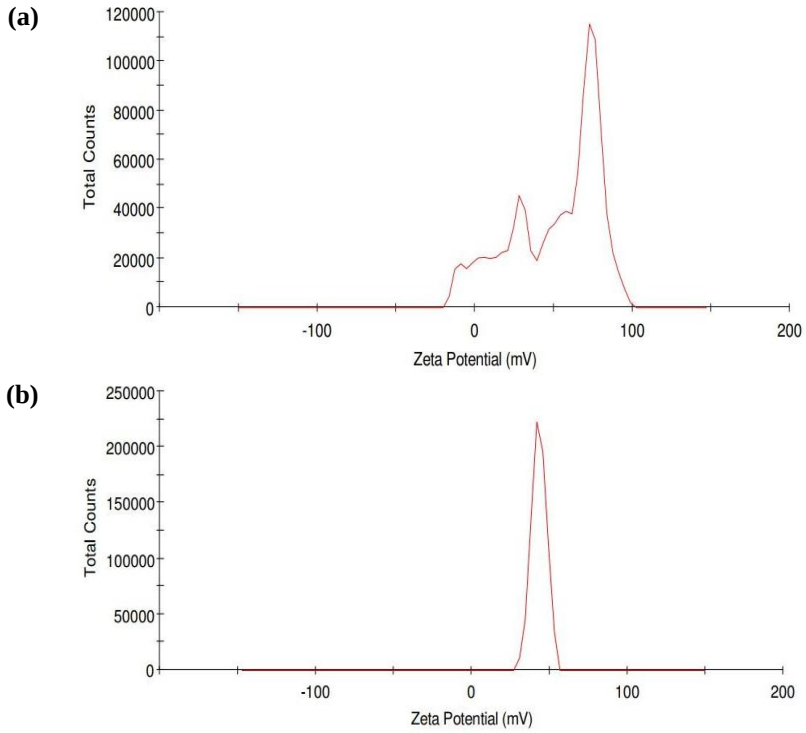


Figure 6. Zeta potential of (a) MAEL-CSNPs and (b) MAEM-CSNPs.

3.8. Mosquito Larvicidal Activity Chitosan Nanoparticles

Among MAEL-CSNPs and MAEM-CSNPs, lower LC50 and LC90 values were observed in MAEL-CSNPs (2.99 and 6.94 µg/mL, respectively). No mortality was found in the negative control (Table 3).

Table 3. Larvicidal activity of MAEL-CSNPs and MAEM-CSNPs against *An. stephensi* after 24-hour exposure.

Treatment	C [µg/mL]	Larval and pupal mortality [%]	LC_{50} (LC_{90}) [µg/mL]	LCL of LC_{50} (LC_{90})	UCL of LC_{50} (LC_{90})	Regression	χ^2 (*DF*=4)
MAEL-CSNPs	2	4.4 ± 0.54[a]	2.99 (6.94)	0.13 (5.46)	4.351 (11.409)	$y = -0.974 + 0.325\ x$	1.855 *n. s.*
	4	6.0 ± 1.22[b]					
	6	7.2 ± 0.83[c]					
	8	10.0 ± 0.0[d]					
	10	10.0 ± 0.0[e]					
MAEM-CSNPs	2	3.6 ± 1.14[a]	3.721 (8.29)	0.854 (6.59)	5.170 (13.477)	$y = -1.042 + 0.280\ x$	0.744 *n. s.*
	4	5.2 ± 1.30[b]					
	6	6.8 ± 0.83[c]					
	8	8.6 ± 0.54[d]					
	10	10.0 ± 0.0[e]					

Note. Negative control nil mortality; C, concentration of MAE encapsulated CSNPs; LC_{50}, lethal concentration 50, referring to the concentration of a substance that is lethal to 50% of the population; LC_{90}, lethal concentration 90, referring to the concentration of a substance that is lethal to 90% of the population; LCL, lower confidence limit; UCL, upper confidence limit; χ^2, chi-square value; DF, degrees of freedom.

3.9. Zebrafish Embryo Toxicity assay

Based on the *in vitro* drug release capability and larvicidal activity, MAEL-CSNPs were selected and subjected to toxicity analysis. The zebrafish eggs were treated with control-MAE and MAEL-CSNPs at the 10 µg/mL concentration. At the beginning of the test (0 h), the eggs are visible as a simple spherical structure. The developmental stage was observed significantly from the 6th hour onwards, and the complete larval maturation stage was found at 48 h. The 6th hour represents the sidewise elaboration of the yolk area, and the 12th hour denotes the anterior area with simple eyes (Figure 7).

Figure 7. Representative images of zebrafish embryos exposed to control and MAEL-CSNPs.

The abdomen and spine were seen during the 24th hour. The mature larvae were only visible on the 48th hour. There is no yolk sac oedema, spine deformation or pericardial malformation during the entire observation time of MAE and MAEL-CSNPs. The 24th and 48th hours of zebrafish larvae represent healthy developments in control, and MAEL-CSNPs showed 100% mortality at a 10 µg/mL concentration. The MAEL-CSNPs below 10 µg/mL concentration may act as a potent larvicide without harming non-targeted organisms.

4. Discussion

Many valuable drugs have been developed using various parts of the medicinally significant plants. The herb *A. absinthium* was also mentioned in ancient Egyptian times as being active against vermin, a common word for vector pests [28], and markedly exhibited strong larvicidal activities toward mosquitoes [29]. It has also been used for centuries as a moth repellent and common pesticide against slugs and snails [30]. The larvicidal bioassays of this investigation revealed the larvicidal potential of various solvent-based aerial part extracts of *A. absinthium*. Among the different extracts, the methanolic extract showed the highest mortality percentage (100%) and the lowest LC_{50} (1.170 mg/L) and LC_{90} (1.819 mg/L), respectively. Larvicidal activities of leaf ethanolic (92 ± 2) and methanolic (68 ± 2) extracts of *A. absinthium* against *Aedes aegypti* were reported [16].

Chitosan nanoparticles were prepared by the ionic cross-linking between the protonated amino groups of the chitosan chains and the phosphate groups of TPP. Chitosan nanoparticles (L-CSNPs and M-CSNPs) were dissolved in different concentrations of MAE, such as 10, 20, 30, 40 and 50 µg/mL, and formed MAEL-CSNPs and MAEM-CSNPs. Among five different concentrations (10, 20, 30, 40 and 50 µg/mL), both MAEL-CSNPs and MAEM-CSNPs showed higher entrapment efficiency, i.e., 64.10% and 59.34%, respectively, at 10 µg/mL. The *in vitro* drug release percentage was higher in MAEL-CSNPs (86.6%) compared to MAEM-CSNPs (80.0%). The drug release percentage was found to be pH dependent. In an acidic medium, an elevation in the ionisation of protonated amine groups causes the bulging of chitosan nanoparticles, which results in more diffusion of methanolic extract from encapsulated nanochitosan [31].

It was found that the *in vitro* MAE release from L-CSNPs and M-CSNPs can be accurately described by Higuchi's equation, as the plots created using this model show the highest linearity (R^2 = 0.978 and 0.893, respectively), followed by zero order (R^2 = 0.952 and R^2=0.888, respectively). The Higuchi model describes the drug discharge from an insoluble matrix as the square root of a time-dependent process based on Fickian diffusion. Moreover, it defines the constant release of a drug from a drug delivery system as involving both dissolution and diffusion. Zero-order kinetics indicates the course of constant drug release from a drug delivery system. If the system follows this mechanism, the drug concentration in the blood remains constant throughout the delivery [32, 33]. As given in the literature, the decrease in intensity of the given bands is due to the removal of acetyl groups during the deacetylation process of chitin [34]. The presence of C–H rocking at 1379 and 1379 cm^{-1} for MAEL-CSNPs and MAEM-CSNPs, respectively, in the FTIR spectra indicates the incorporation of alkanes being a constituent of the plant extract (MAE) [35]. XRD analysis of MAEL-CSNPs and MAEM-CSNPs shows differences in intensity and peak position [36]. The amorphous nature of CSNPs was observed for both samples in XRD analysis. This nature results from the destruction of the crystalline structure of chitosan after crosslinking with TPP [37–39].

MAEL-CSNPs and MAEM-CSNPs presented a homogenous topographic surface, but MAEL-CSNPs showed smaller nanoparticles than MAEM-CSNPs [40]. In zeta potential analysis, MAEM-CSNPs showed a higher charge, i.e., +43.2 mV, than MAEL-CSNPs, i.e., +30.8 mV. The zeta potential of nanoparticles increased with the increase in molecular weight of chitosan [41]. Nanoparticles with a surface charge of +30mV have been revealed to be stable, as the surface charge is adequate to avoid aggregation of the particles [42].

Mosquitoes are the potential vectors of various diseases such as dengue, malaria and brain fever. Effective control of these diseases is possible through early diagnosis and proper treatment. The use of synthetic insecticides can increase mosquito resistance and environmental pollution. Therefore, the development of effective and eco-friendly botanical insecticides is needed. Nanotechnology is a promising field of research with broad applications in vector control. Nanoencapsulation strategies can enhance the efficiency of plant extracts or plant-derived compounds by protecting them from degradation and providing a controlled release rate. Among the polymers used for encapsulation, chitosan stands out because of its various biological properties, such as non-toxicity, biocompatibility and biodegradability [43].

In this study, encapsulated chitosan nanoparticles (MAEL-CSNPs and MAEM-CSNPs) were tested for their mosquito larvicidal activity. Among various treatments, MAEL-CSNPs exhibited lower LC_{50} and LC_{90} compared to MAEM-CSNPs. Our results are similar to the results of Paula *et al.* [44], who found the highest larvicidal effect against *Stegomyia aegypti* larvae by using $1.8 \cdot 10^5$ g/mol molecular weight chitosan and angico cum nanoparticles loaded with essential oil of *Lippia sidoides*.

To determine the nanoparticle toxicity against non-targeted organisms, zebrafish embryos were treated with control-MAE and MAEL-CSNPs at a 10 µg/mL concentration. The 24 and 48 hours of zebrafish larvae represent healthy developments in control, and MAEL-CSNPs showed 100% mortality at a 10 µg/mL concentration. The MAEL-CSNPs below 10 µg/mL concentration may act as a potent larvicide without harming non-targeted organisms. A similar study was made by Chou *et al*. [45], who used low molecular weight chitosan-TPP NPs at different concentrations (10 - 100 µg/mL) to treat zebrafish larvae and found that increasing the concentration of NPs decreases the survival rate [45].

5. Conclusions

Encapsulation of herbal extracts with chitosan nanoparticles or chitosan-plant extract complexes is a worthwhile approach in developing eco-friendly larvicides. Based on the present investigation, it can be concluded that the methanolic extract of the aerial parts of *A. absinthium* was found to be a potent larvicidal extract against *An. stephensi* larvae. This larvicidal potential was further confirmed by *in vitro* drug release kinetics and the larvicidal activity of the chitosan-plant extract complex. Toxicity analysis of MAEL-CSNPs using zebrafish supports its larvicidal effect against *An. stephensi* larvae if the NPs are used below a 10 µg/mL concentration. Overall, the findings are helpful in the development of more potent, biodegradable and nontoxic herbal-based larvicide against the malaria vector. However, further investigations are necessary to determine the mode of the individual chemical compound's actions, its effects on non-targeted organisms and field evaluation.

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