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Eco-friendly larvicidal agent from combined plant extracts encapsulated chitosan nanoparticles to combat dengue mosquitoes

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Abstract

Considering reduced environmental impact and high specificity in targeting vector organisms, the use of plant-based insecticides for dengue mosquito control is crucial in dengue disease management. We investigated the larvicidal efficacy of hot water, cold water, and water-acetone extracts of combined plant extracts of *Capsicum frutescens* (Naimiris) and *Allium sativum* (Garlic) against *Aedes aegypti* and *Aedes albopictus* larvae. The plant extract encapsulated chitosan nanoparticles were also assessed for larvicidal potential. Chitosan nanoparticles were synthesized via the ionic gelation method, employing an optimized formulation comprising 2.5% chitosan and 1.5% sodium tripolyphosphate, with stirring at 1500 rpm. The most effective plant extract was encapsulated into chitosan nanoparticles, subsequently packaged into commercially viable sachets. The water-acetone extract showed the highest larval mortality, resulting the lowest LD_{50} values (2051.01 and 1097.69 mg L^{-1} after 24 and 48 hours of exposure, respectively), while showing the significant synergistic activity (CI < 1). Nanoparticle characterization was conducted using Scanning Electron Microscopy and Fourier Transform Infrared Spectroscopy, revealing a nanoparticle diameter ranging from 350 to 450 nm. Approximately 96.10 ± 2.77% of combined plant mixture's water-acetone extract was successfully encapsulated within nanoparticles, with 90% of encapsulated plant extract released into the medium over a 14-day period. Semi-permeable sachet packets containing 6 g of encapsulated chitosan nanoparticles showed strong larvicidal activity for 10 days, with shelf life up to six months. These findings suggest that chitosan nanoparticles encapsulating a combination of *Capsicum frutescens* and *Allium sativum* extracts hold promise as eco-friendly larvicides against dengue vectors.

Keywords: *Allium sativum, Capsicum frutescens,* Chitosan nanoparticles, Dengue, Encapsulation

1. Introduction

Mosquitoes are well known viral vectors that transmit several destructive diseases such as Malaria, ZIKA, Dengue, Chikungunya, Yellow fever, and Japanese encephalitis for humans (Raveen, et al., 2017). Among the vector-borne diseases, dengue is considered one of the most dangerous viral diseases transmitted by *Aedes aegypti* and *Aedes albopictus* (AhbiRami, et al., 2014; Hajra, et al., 2015). Southeast Asia and Western Pacific regions are the most affected by dengue though it is prevalent in more than 100 countries. Moreover, WHO estimated about half of the world population is now at risk of dengue with an estimated 100-400 million infections occur each year (WHO, 2022). Effective vaccines are not available except Dengvaxia which is mostly recommended for people who are already influenced by dengue fever (Manna, et al., 2022). Hence, the early detection and access to proper medical care and the control of vector mosquitoes have been recommended to control dengue as the disease is a burden to the national healthcare sector especially in developing countries.

In recent decades the mosquito population has been drastically increased due to urbanization and climate change (Biswal, 2023). As a result, dengue infection is considerably high in urban areas (Kumar, et al., 2017). Mosquito control programs have been introduced to control the vector mosquito population which is mainly centred with the use of chemical insecticides (Karunaratna, et al., 2018). Eliminating larval habitats, reducing life-span of adult mosquitoes, biological control using larvae predators, reduction and preventing human-mosquito contact, are the other common mosquito controlling methods employed worldwide (WHO, 2022). The control approach which targets the larvae population is relatively effective and less challenging due to the restricted habitats, less movements, and the relatively less amount of larvicide requirement (AhbiRami, et al., 2014; Gutierrez, et al., 2014). Nowadays, a plethora of synthetic chemicals, belongs to Organophosphates, Organochlorines, Carbamates, and Pyrethroids chemical groups have been commonly used for mosquito control (Karunaratna, et al., 2018; Fernando, et al., 2020; Pilaquinga, et al., 2019). However, these insecticides have been identified as high risk for the environment due to non-target effects, biological magnification, and the persistent in the environment. In addition, prolonged use of these have resulted in the development of resistance against mosquitoes (Gutierrez, et al., 2014; Karunaratna, et al., 2018). Therefore, much attention has been paid for developing safer and efficient methods to control mosquitoes and identification of natural larvicidal agents is one such method that has taken top priority in the development of novel larvicides.

Plants are a good source of bioactive compounds. Various phytochemicals and essential oils which are naturally present in plant extracts have a specific ability in killing mosquito larvae (Kour & Riat, 2021). The plant extracts are mostly target specific and less harmful to other organisms and the environment. Further, the plant-based mosquito larvicides are known for less resistance development (Raveen, et al., 2017; Pilaquinga, et al., 2019). The plants used in this study are *Capsicum frutescens* fruits (Naimiris) and cloves of *Allium sativum.* Among the many important bioactivities, both plants showed mosquito larvicidal properties (Anand, et al., 2018; Vasconcelos, et al., 2014; Kasim, et al., 2019; Kala, et al., 2010; Muturi, et al., 2018). However, no reports were found for the larvicidal properties of the combined extracts of *Capsicum frutescens* fruits and *Allium sativum*. In this research synergistic properties of combined plant extract were utilized to achieve the maximum efficiency and larvicidal potency.

Nevertheless, practical use of these plant extracts itself in water-based resources where the mosquito larvae are produced is problematic due to the decomposition of the active ingredients and wastage. Hence, encapsulation of active plant extracts to water insoluble nano carriers can be applied in order to obtain the highest efficiency (Mattu, et al., 2013; Kanddestani, et al., 2020). Encapsulation emerged to decrease their sensitivity to degradation. Due to outstanding properties of biopolymer based nanoparticles, they have been used as an encapsulant to protect bioactive compounds by loading inside and thus to protect from degradation by direct exposure to adverse conditions such as light, heat, oxygen and microbial infections (Kashyap, et al., 2015).

Chitosan is a biopolymer that is derived from the deacetylation of chitin have unique properties including biocompatible, nontoxic, biodegradable, good bioadhesive properties, ease in preparation, control release of bioactive compounds, etc (Barbosa, et al., 2011; Alonso & Goycoolea, 2020). Chitosan nanoparticles have an ability to form crosslinks with bioactive compounds, therefore bioactive compounds can be encapsulated into chitosan nanoparticles to reach their optimum functions and the stability (Hajra, et al., 2015). In the present study, crude extracts of mixture of fruits of *Capsicum frutescens* (Naimiris), and cloves of *Allium sativum* are encapsulated into chitosan nanoparticles in order to synthesize eco-friendly highly larvicidal active product while minimizing the wastage of active ingredients.

2. Materials and Methods

Chitosan (low molecular weight and deacetylation degree of 90%), Sodium Tripolyphosphate (STPP) and Polysorbate 80 (Tween 80) were purchased from Sisco Research Laboratories Pvt. Ltd, India. All other chemicals and solvents that were not mentioned above were analytical grade and used without further purification.

2.1 Preparation of plant extracts

Healthy and fresh, green *Capsicum frutescens* fruits (Naimiris) and *Allium sativum* (garlic) were collected from the Kandy district, Sri Lanka. The collected garlic was peeled off and all plant materials were washed thoroughly with running tap water followed by distilled water. Subsequently, an equal amount (50 g) of plant material was weighed and cut into small pieces and were ground into a fine paste using the blender. Three solvent systems: hot water, cold water and water: acetone (1:1) were used for the extraction of active compounds. Hot water extracts were prepared using refluxing for 3 hours. Cold water and water: acetone (1:1) extracts were prepared by maceration using a bottle shaker (24 hours x 3). Subsequently, extracts were filtered through no 1 Whatman filter papers. Following the filtration, and samples were concentrated using rotary vacuum evaporator to obtain crude plant extracts. Trace amount of water was removed by freeze drying. Dried extracts were stored at 4 °C until further use.

2.2 Larvae cultures and larvicidal bioassay

The larvae of *Aedes aegypti* and *Aedes albopictus* were collected from different breeding sites such as water ponds, wastewater streams, discarded jars, earthen pots, bamboo trunks, and rainwater gutters using plastic cups and larvae droppers. The larvae were identified to genera level in the field by observing the resting angle to the water surface and size and shape of the respiratory siphone. The larvae were reared in the laboratory at 25 ± 2 °C temperature, 80 ± 10% relative humidity with photoperiod of 12 hour light and 12 hour dark conditions. They were fed once a week with a mixture of dog biscuits and brewer's yeast in 1:1 ratio as fine powder. The identification was confirmed with the taxonomic features of the emerged adults.

10000 mg L^{-1} stock solution for each plant extract was prepared and the test solutions were prepared diluting as 5000, 2000 and 1000 mg L^{-1}. This step was followed for plant crudes obtained from hot water, cold water and acetone: water. Twenty larvae of 3rd instar of *Aedes* larvae were transferred into beakers containing 90 mL of distilled water. And then 10 mL of each test solution was added to the beaker slowly through walls. Percentage larval mortalities were calculated after a 24 and 48 hour exposure period. The control experiment was set up with the distilled water. Moribund larvae that are incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed were considered as dead larvae. In addition, the individual larvicidal effects of *C. frutescens* and *A. sativum* were also tested using the most effective solvent obtained from above results.

$$\%\, mortality = \frac{number\ of\ dead\ larvae}{total\ number\ of\ larvae\ exposed} \times 100$$

2.3 Determination of synergistic effect of plant mixture using combination index (CI)

Combination index measures the degree of drug interaction, and its formula is the sum of the ratio of the dose of individual drug in the compound to the dose when used alone when the combination and compound produce 50% efficacy. Synergistic activity was calculated using the combination index (CI) method described by Ting-Chao Chou (2018). If, CI= 1, indicates additive effect in the absence of synergism or antagonism. CI <1, indicates synergism while CI >1, indicates antagonism. The synergism of the activity was calculated using following the formula described by Chou (2018).

$$Combination\ Index\ (CI) = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} + \frac{(D)3}{(Dx)3} + \cdots + \frac{(D)n}{(Dx)n}$$

$(D)_1$ – concentration of plant extract 1 in combination $[(D)_1 + (D)_2 + (D)_3]$ kill 50% larvae.

$(Dx)_1$- concentration of plant extract 1 alone that kill 50% larvae.

2.4 Synthesis of chitosan nanoparticles

Chitosan nanoparticles were synthesized using ionotropic gelation method following a modified method by Katuwavila et al (2016) and optimized to obtain nano range particle size. In brief, 1.0, 1.5, 2.0, 2.5 and 3.0% chitosan concentration series, 0.25, 0.5, 1.0, 1.5 and 2.0% TPP concentration series, 1000 and 1500 rpm stirring speeds were used to optimize processes. When changing the one parameter, other parameters were kept constant.

Chitosan solutions were prepared by dissolving with 1% acetic acid using a magnetic stirrer overnight at 250 rpm. Then, chitosan solutions were filtered through 0.45 µm pore size filter papers and pH were adjusted to 4.8 using NaOH. Sodium tripolyphosphate (TPP) solutions were prepared by dissolving in distilled water and were filtered through Whatman filter paper. pH of TPP solutions were adjusted to 5.2 using HCl. 20 mL of chitosan solution was stirred with 0.5 mL of tween 80 at 60 °C for two hours to obtain a homogeneous mixture. Then, 20

mL TPP solution was added to the chitosan solution slowly dropwise manner while stirring at room temperature over one hour. Stirring was continued for another half an hour and the resultant suspension was centrifuged at 10000 rpm for 20 minutes. Subsequently, supernatant was discarded and solid mass obtained was washed with distilled water several times followed by centrifugation. Then, the samples were dried at ambient temperature using vacuum oven for five hours. Plant extract encapsulated chitosan nanoparticles were prepared following the same protocol however, 1 g of plant crude was added before the addition of TPP into the solution.

2.5 Characterization of chitosan nanoparticles

The particle size and size distribution of the nanoparticles were performed by particle size analyser (CILAS-NANO D5). Size and the surface morphology of the nanoparticles were studied using Scanning Electron Microscopy (EVO LS 15) by selecting voltage of 20 kV. UV-Vis absorption spectra were recorded over wavelengths from 200-1100 nm by SHIMADZU UV Spectrophotometer. Fourier Transform Infrared Spectra were taken with KBr pellet using FTIR instrument (JASCO-6700).

2.6 Determination of encapsulation efficiency (EE)

Encapsulation efficiency was determined according to the method described by Katuvawila et al (2016). In brief, the amount of incorporated plant extract in the nanoparticles was determined by measuring the remaining absorbance of plant extract in the supernatant after centrifugation using UV-Vis spectrometer at 672 nm. The calibration curve was obtained for a series of standard plant crude concentrations to obtain the unknown concentration of free plant extract. Encapsulated concentrations of plant extracts were determined using the absorbance values from the calibration curve and the percent encapsulation efficiency was calculated using the following equation. Encapsulation experiment was repeated three times and average data were recorded.

$$EE\ (\%) = \frac{total\ extract\ amount - free\ extract\ amount}{total\ extract\ amount} \times 100$$

2.7 Determination of releasing efficiency (RE)

6 g of plant extract encapsulated chitosan nanoparticles were placed into a dialysis bag, and it was placed in a beaker containing 100 mL distilled water at room temperature. During the releasing test, 3 mL of release medium was taken at predetermined time intervals for absorbance measurements using UV-Vis spectrophotometer at 672 nm. When 3 mL of release medium was taken, same volume of fresh distilled water was added to the solution to maintain the final volume of the releasing setup. Finally, the released amount was determined using the calibration curve and the following equation. Releasing experiment was repeated three times and average data were recorded.

$$RE\ (\%) = \frac{released\ amount\ of\ plant\ extract}{total\ amount\ of\ plant\ extract} \times 100$$

2.8 Larvicidal activity of plant extract encapsulated chitosan nanoparticles

1.0, 2.0, 4.0, 5.0, and 6.0 g of plant extract encapsulated chitosan nanoparticles were filled into empty semi permeable sachet packets and were dipped in a beaker containing 20 of 3rd instar of *Aedes* larvae in 100 mL distilled water. Larvae mortality was calculated after a 24 and 48

hour exposure period. 6.0 g of empty chitosan nanoparticles (without plant extract) were used as the control. Three replicates were carried out for each concentration of chitosan nanoparticles and larval mortalities were calculated.

Moreover, the larvicidal activity of one sachet packet containing 6 g of plant extract encapsulated chitosan nanoparticles was tested for the same time period that the encapsulated plant extract showed the maximum released to the medium. 10 *Aedes* larvae were added on each day to the medium which include 100 mL distilled water and the sachet packet. After that, percent larvae mortality was calculated each day.

$$\%\, mortality\ = \frac{number\ of\ dead\ larvae}{total\ number\ of\ larvae\ exposed} \times 100$$

2.9 Study of the shelf life

Shelf life of plant extract encapsulated nanoparticles was determined at three different temperatures; room temperature (27 °C), refrigerator (4 °C) and at 40 °C in an oven. In the accelerated shelf-life method, one week at 40 °C in an oven is approximated to one and half months (Richards, et al., 2014).

6.0 g of plant extract encapsulated chitosan nanoparticles were covered with aluminium foil and were placed in room temperature (27 °C), refrigerator (4 °C), and the oven (40 °C) for 1, 2, 3, and 4 weeks. Larvicidal activity was tested each week and larval mortalities were calculated. Three replications were carried out for above three temperature conditions.

Data Analysis

Larval mortality percentages resulting from bioassay experiments, along with encapsulation efficiency and releasing efficiency, were quantified using standard equations outlined in the respective methodology section. The concentrations of unknown plant extracts were determined through a standard calibration curve constructed using known concentrations of crude plant extracts. LD_{50} values were obtained from the curves derived from Origin software. The synergistic effect of the combined plant mixture was assessed through the combination index, calculated by summating LD_{50} values obtained from both individual and combined plant extracts. Verification of plant extract encapsulation into chitosan nanoparticles was established through the identification of specific IR bands derived from Fourier Transform Infrared Spectroscopy (FTIR).

3. Results

3.1 Larvicidal activity of combined and individual plant extracts

The results of the larvicidal activity of hot water, cold water, and water: acetone (1:1) extracts of combined plant mixture of *Capsicum frutescens* and *Allium sativum* against the third instar larvae of mosquitoes of the genus *Aedes* is shown in figure 1. The highest larval mortalities were observed in the water: acetone extracts (100% after 24 hrs) while cold water extract showed 90% and 100% respective mortalities after 24 hours and 48 hours exposure period. 10000 mg L^{-1} concentration of those extracts showed the highest activity against larvae.

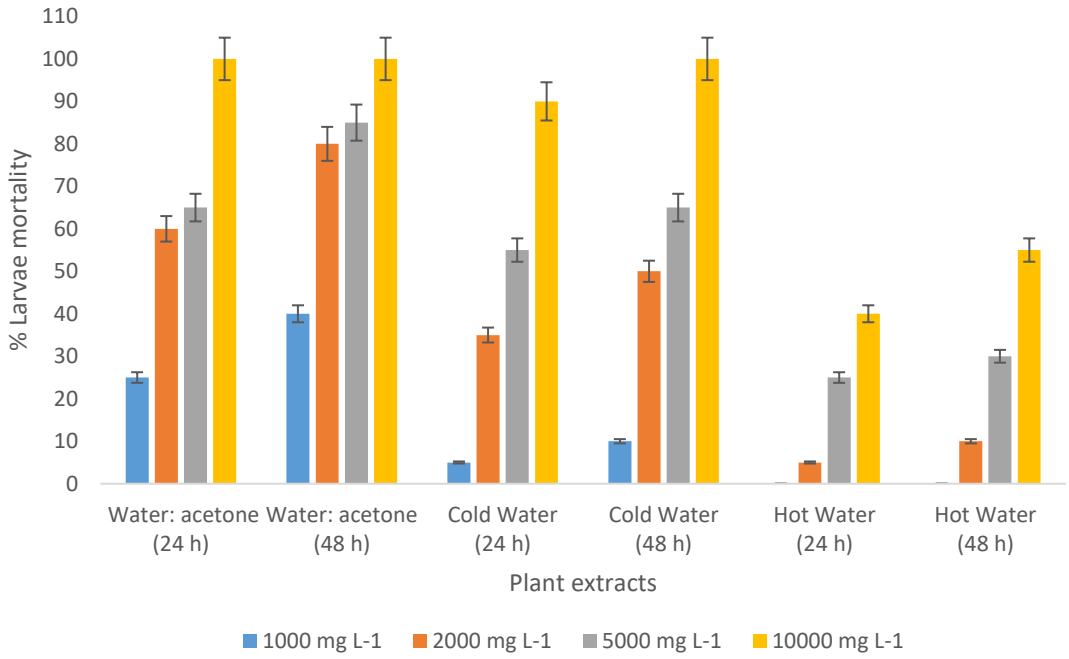

Fig. 1 Percentage mortalities of *Aedes* larvae obtained from bioassay experiments conducted with combined plant mixture for hot water (H.W), cold water (C.W) and water: acetone (W:A) extracts after 24 and 48 hours exposure period

Since the highest activity was obtained for the water: acetone extract, individual extracts of each plant were prepared using water: acetone (1:1) solvent system in order to identify the larvicidal activity and determine synergistic effect on combined plant mixture. While individual extract of green *C. frutescens* showed 70% and 90% larvae mortalities, individual extract of *A. sativum* showed 75% and 85% mortality after 24 and 48 hours respectively (Figure 2).

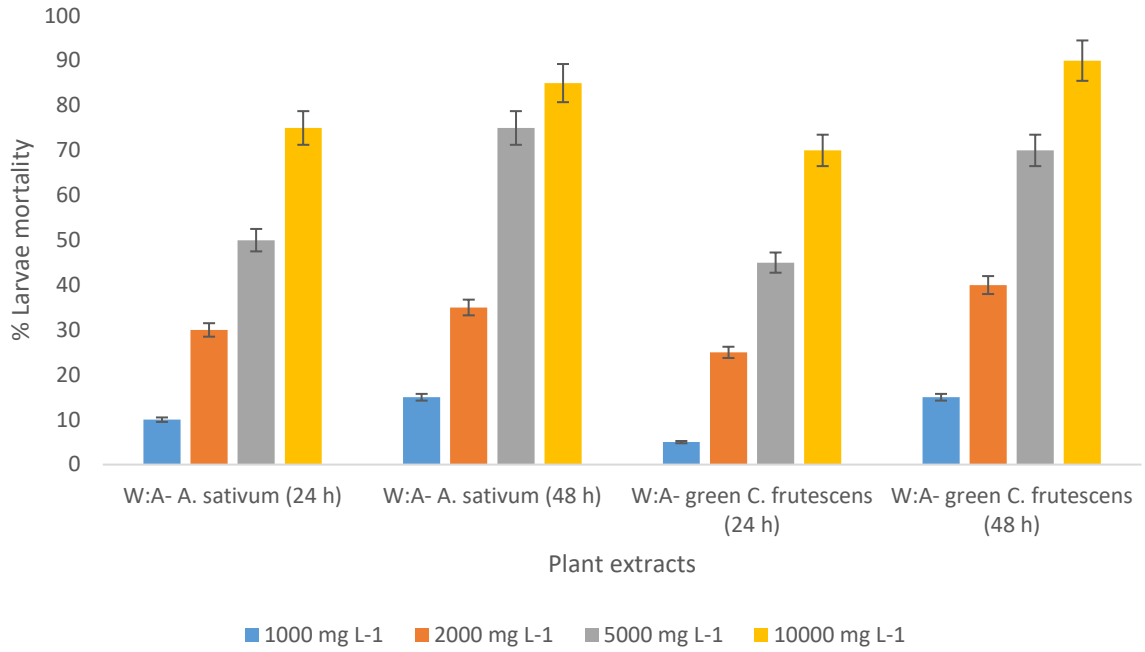

Fig. 2 Percentage mortalities of *Aedes* larvae obtained from bioassay experiments conducted with water: acetone extracts (W:A) of individual plant extracts after 24 and 48 hours exposure period

Table 1 presents the LD_{50} values derived from bioassay experiments. The lowest LD_{50} values of 2051.01 and 1097.69 mg L^{-1} after 24 and 48-hour exposure period was reported by the water: acetone extract. The larvae mortalities were positively correlated with the concentrations of the treatments and the exposure time. LD_{50} values of the individual extract of green *C. frutescens* were 5228.12 and 2819.03 mg L^{-1} for 24 and 48-hours exposure respectively. Similarly, individual extract of *A. sativum* showed LD_{50} of 4353.73 and 2925.95 mg L^{-1} for the respective time periods.

Table 1

LD_{50} values of the Bioassay experiments conducted with hot water, cold water and water: acetone solvent extracts. r and p values ($P<0.05$) represent the results of correlation analysis.

Extraction	Plant mixture	LD_{50} value/ mg L^{-1}		Correlation coefficient (r)	P value
		After 24h	After 48h		
Hot water	Mixture of green *C. frutescens* and *A. sativum*	>10000	9614.49	0.9817	0.0183
Cold water	Mixture of green *C. frutescens* and *A. sativum*	3521.66	2657.93	0.9768	0.0232
Water: acetone	Mixture of green *C. frutescens* and *A. sativum*	2051.01	1097.69	0.9573	0.0427

	Green *Capsicum frutescens*	5228.12	2819.03	0.9905	0.0095
	Allium sativum	4354.73	2925.95	0.9909	0.0091

3.2 Formation of plant extract encapsulated chitosan nanoparticles and characterization

Concentrations and reaction conditions were optimized to obtain the minimum particle size and 2% chitosan and 1.5% TPP concentrations were found to be the best conditions to obtain nanoparticles with the lowest particle size and high porosity. In addition, stirring speed of 1500 rpm was selected as the optimized stirring condition. In this study, optimized particle size in the diameter range of 350-450 nm was obtained from particle size analyser. Average size for optimized formulation was about 390 nm (Figure 3). Figure 4 presents the Scanning Electron Microscopic images of chitosan nanoparticles that are white colour and spherical in shape. Crude plant extract gives a maximum absorption peak at 672 nm in UV therefore, all absorbance measurements were taken with respect to 672 nm (Figure 5).

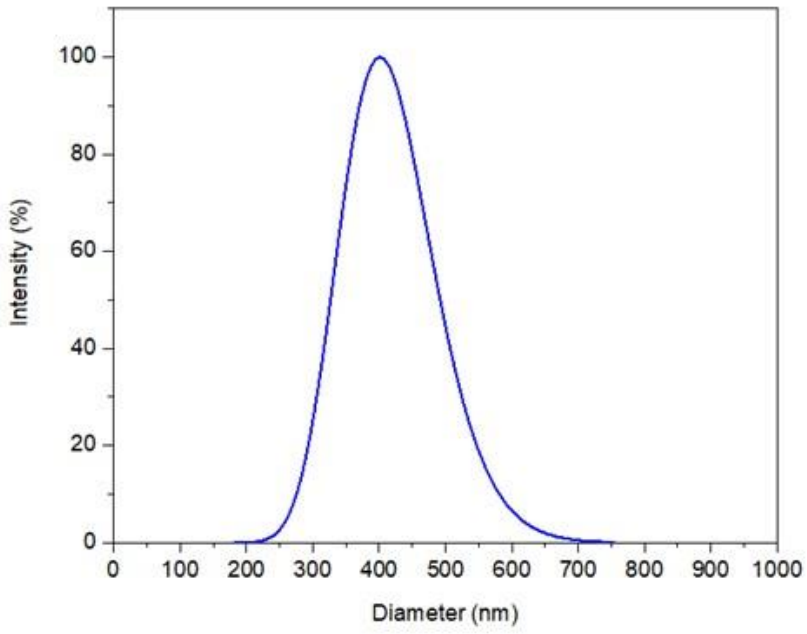

Fig. 3 Particle size distribution of optimized chitosan nanoparticles

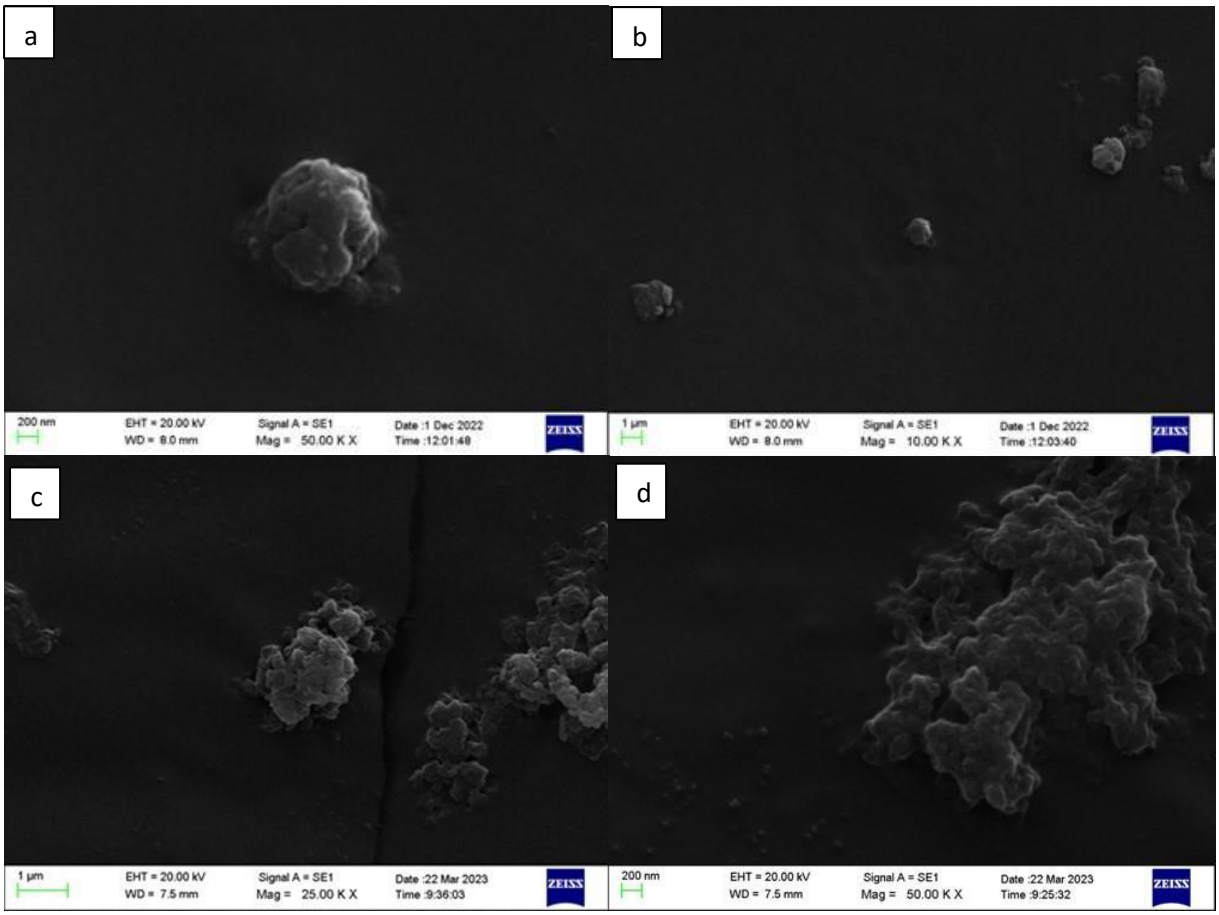

Fig. 4 SEM images of chitosan nanoparticles before the encapsulation of plant crude extract (a and b) and plant extract encapsulated chitosan nanoparticles (c and d)

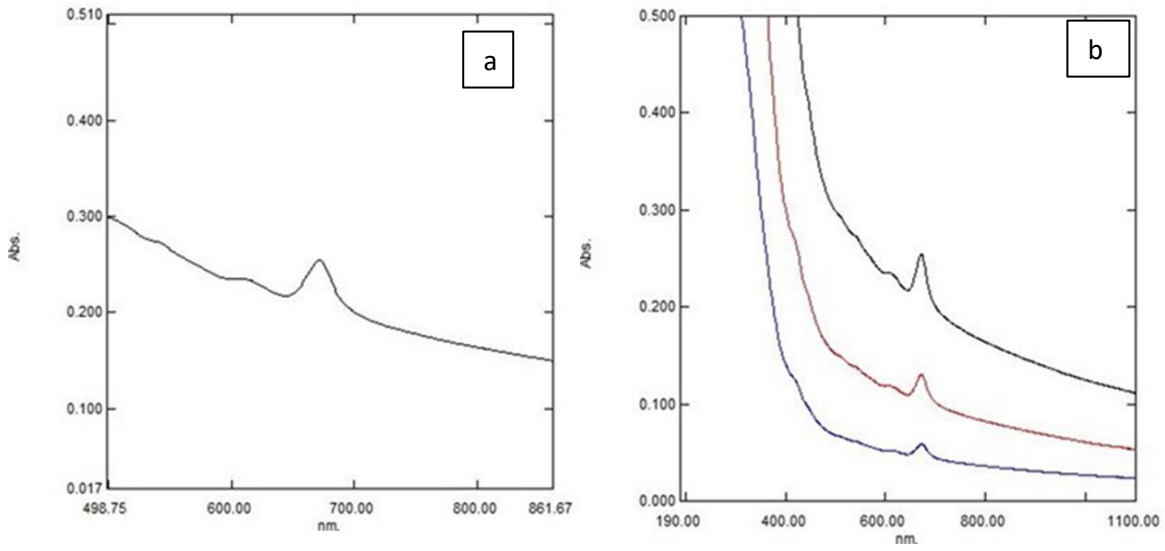

Fig. 5 Absorbance wavelength of plant crude which includes combined mixture of green *C. frutescens* and *A. sativum* (a) and absorbance wavelength of plant crude at different concentrations (b)

Successfully loaded crude plant extract into chitosan nanoparticles was confirmed by FTIR. FTIR spectrums of chitosan, blank chitosan nanoparticles, plant extract, plant extract loaded chitosan nanoparticles, and TPP were shown in figure 6. In the spectra of chitosan, the broad band at 3342 cm^{-1} corresponds to the $–NH_2$ and –OH groups and the bands at 1646 and 1588 cm^{-1} are for –CO stretching and $–NH_2$ bending vibration respectively. During the gelation reaction, as the NH_2 present in chitosan crosslinked with PO_4^{3-} present in TPP, shifting of the broad band at 3342 to 3307 cm^{-1} was observed (Saharan, et al., 2015; Kanddestani, et al., 2020). Presence of the IR bands in plant extracts at 1735, 1424, 1369, 1218, and 1141 cm^{-1} in the plant extract encapsulated chitosan nanoparticles confirmed that the plant extract has successfully encapsulated into chitosan nanoparticles (Ruslan, et al., 2020; Yoksa, et al., 2010).

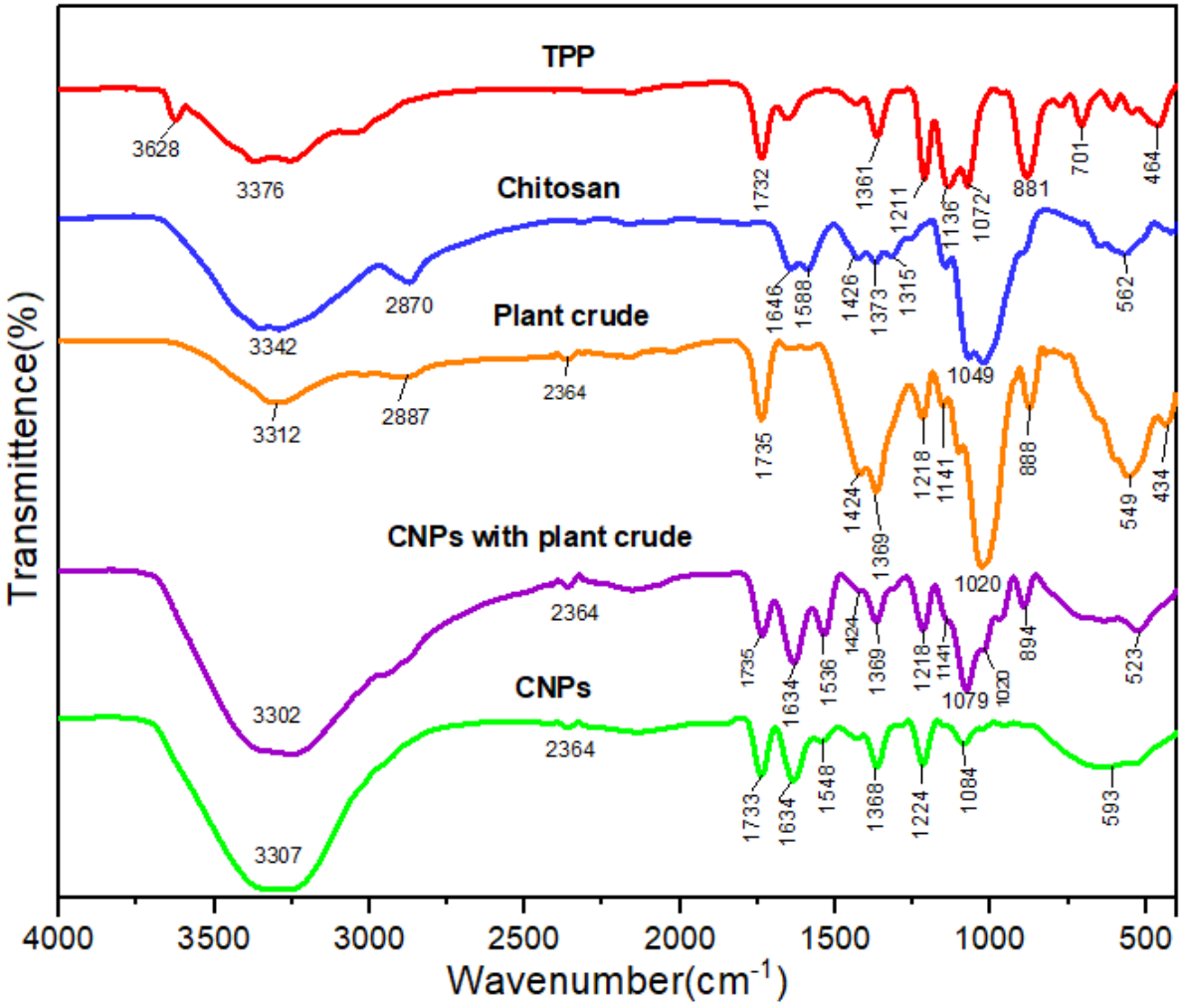

Fig. 6 The FTIR spectra of sodium tripolyphosphate (TPP), chitosan, crude plant extract, Chitosan nanoparticles (CNPs), and plant extract encapsulated chitosan nanoparticles

3.3 Encapsulating and releasing efficiency of plant extract encapsulated chitosan nanoparticles

The highest encapsulation efficiency of chitosan nanoparticles was 96.22 ± 2.06 % for 1000 mg of crude plant extract. 89.19 ± 2.06, 84.35 ± 0.36, 75.38 ± 2.55, and 73.23 ± 2.09 % encapsulated yield obtained for other respective amounts of crude plant extracts (Table 2).

Table 2
Encapsulation efficiency of various amounts of plant crude to determine the optimum amount of plant crude

% chitosan	% TPP	Amount of plant crude/ g	% Encapsulation efficiency
2.0	1.5	0.05	73.23 ± 2.09
		0.12	75.39 ± 2.55
		0.25	84.19 ± 0.36
		0.50	89.19 ± 2.05
		1.00	96.10 ± 2.77

The releasing study and larvae testing experiments were carried out at a neutral pH value which was selected by analysing 20 water samples from the different breeding sites that *Aedes* larvae were living in. Release of plant extract from the chitosan nanoparticles was observed up to 14 days. The cumulative percentage of plant extract released was obtained to be about 90%. When considering the liberation of plant extract from nanoparticles, the initial burst release may be due to fast swelling or diffusion of poorly encapsulated or adsorbed plant extract in the nanoparticles. Also, 36% and 60% of plant extract were released to the medium after 24 and 48 hours respectively. The liberation of plant extract is initially rapidly increased and then gradually decreased until 162 hours (seven days) and then saturated the medium until 14 days (Figure 7).

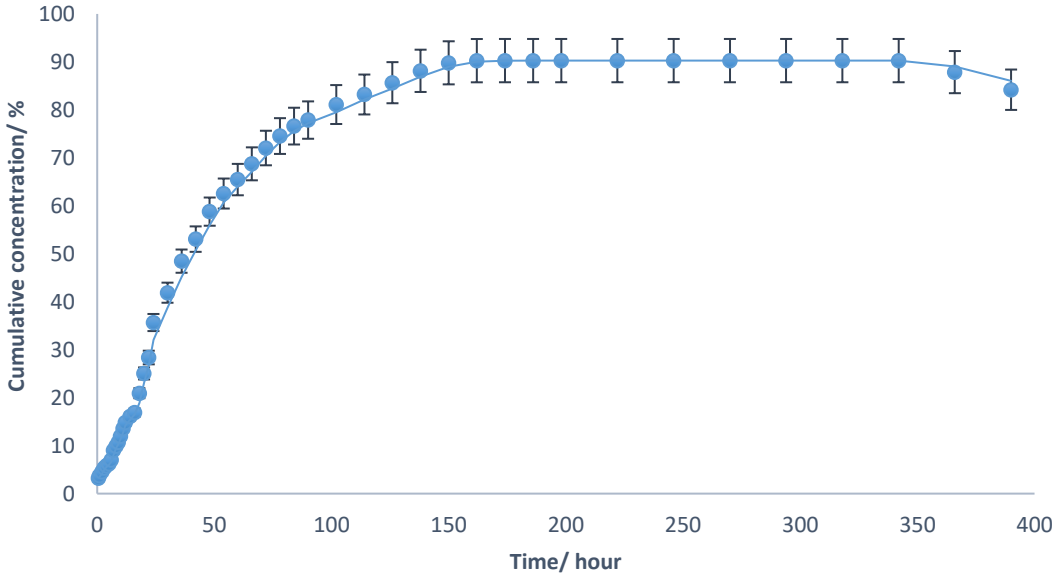

Fig. 7 Percent cumulative release of plant extract encapsulated chitosan nanoparticles with time at neutral pH

3.4 Larvicidal activity of plant extract encapsulated chitosan nanoparticles

Figure 8 presents the larvicidal activity of plant extract encapsulated chitosan nanoparticles. Results revealed that larvae mortality is concentration and time dependent. 100% larvae mortality in 24 hours is observed for 6 g of plant crude encapsulated chitosan nanoparticles. LD_{50} values of plant extract encapsulated chitosan nanoparticles are 2.31 and 1.40 g for 24 and 48 hours exposure period to *Aedes* larvae respectively (Figure 9).

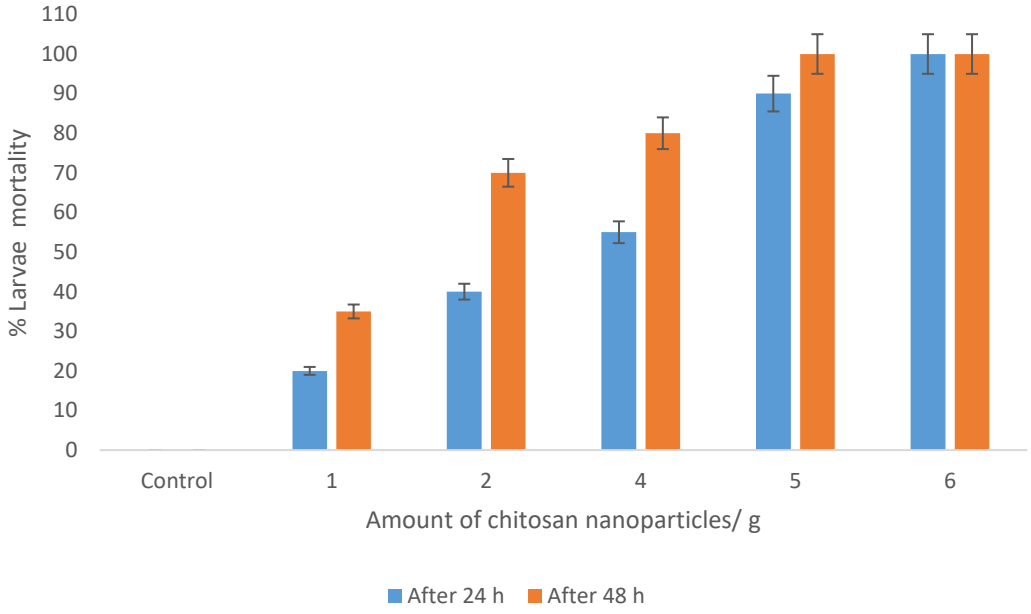

Fig. 8 Mean % larval mortalities obtained from larvicidal assays of plant extract encapsulated chitosan nanoparticles after 24 and 48 hours treatment

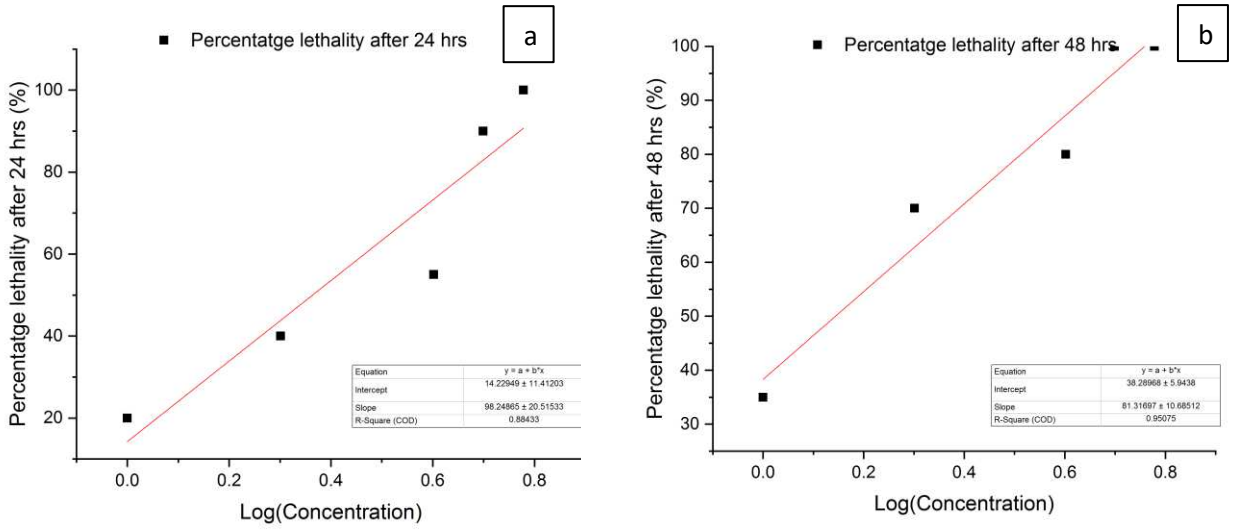

Fig. 9 The graphs used to determine the LD_{50} values of plant extract encapsulated chitosan nanoparticles after 24 h (a) and 48 h (b) treatment

Figure 10 summarizes the results of the larvicidal bioassay done with the sachet packet containing 6 g of plant extract encapsulated chitosan nanoparticles for 14 days. The results revealed that 100% larvae mortality up to 9 days and gradual reduction of the larval mortalities. Only 10% larval mortalities were observed in day 12.

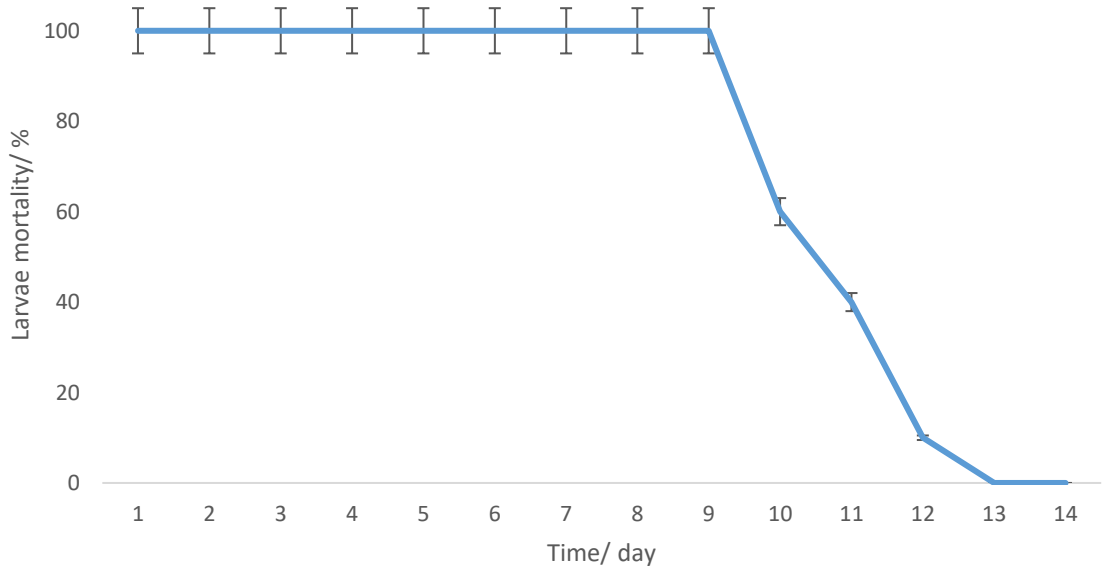

Fig. 10 Mean % larval mortalities obtained from larvicidal assays of plant extract encapsulated chitosan nanoparticles over 14 days of time period

3.5 Study of the shelf life of plant extract encapsulated chitosan nanoparticles

Figures 11 and 12 show the outcomes of the experiments aimed at determining the shelf life of chitosan nanoparticles encapsulating plant extracts, with a focus on long-lasting efficiency. Throughout the experiments, samples stored at both refrigerated and room temperatures exhibited 100% larval mortality. Conversely, the sample subjected to oven storage maintained 100% mortality for a duration of two weeks. Notably, beyond this period, a decrease in larvicidal activity was observed, attributed to the impact of heat on the encapsulated plant extract. However, the reduction in mortality, though evident after two weeks, did not reach a statistically significant level when compared to the initial 100% mortality rate.

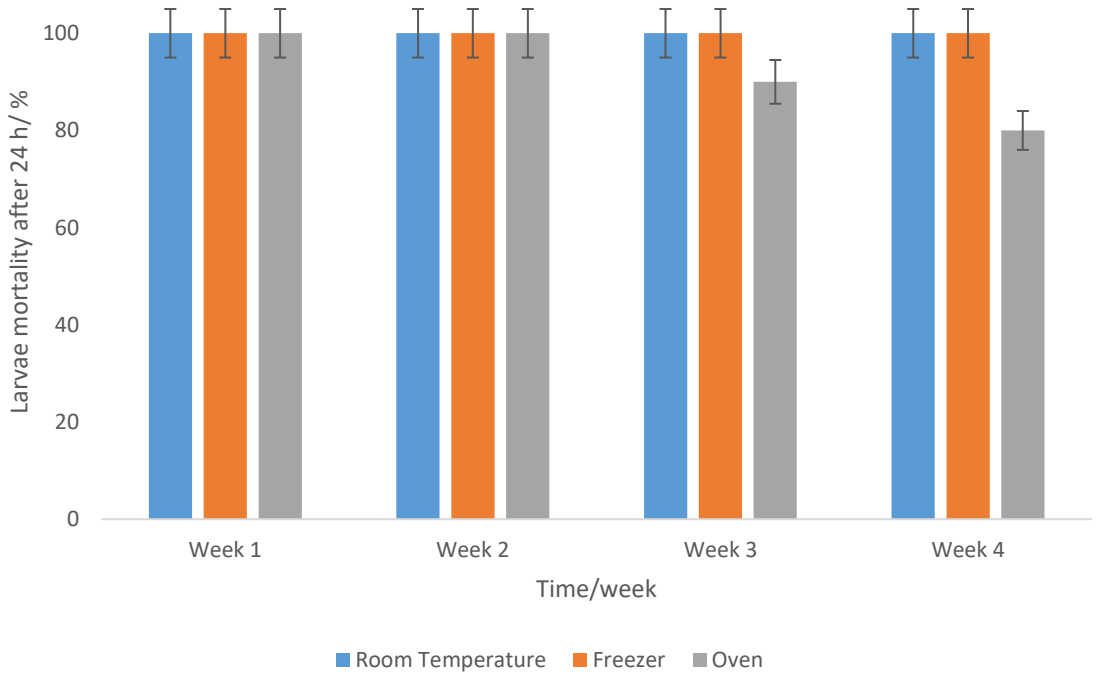

Fig. 11 Mean % larval mortalities obtained from a larvicidal assay of 6 g of plant extract encapsulated chitosan nanoparticles after 24 hours exposure period for the determination of shelf life

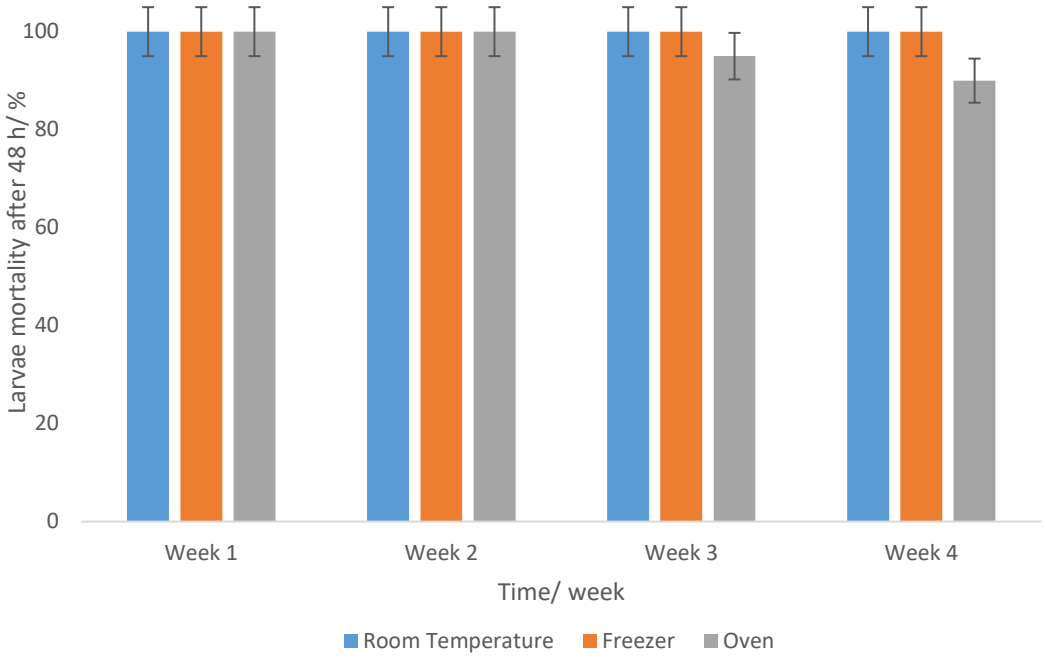

Fig. 12 Mean % larval mortalities obtained from larvicidal assay of 6 g of plant extract encapsulated chitosan nanoparticles after 48 hours exposure period for the determination of shelf life

4. Discussion

The development and production of safer, eco-friendly, cost-effective, biodegradable, more efficient, and target-specific plant-based larvicides as a simple and sustainable method of mosquito control have become one of the most effective alternative approaches for controlling mosquito population (Sukuma, et al., 1991; Shaalan, et al., 2005; Dubey, et al., 2008). In this research natural larvicidal agent using plant crude extracts as an alternative larvicide for synthetic chemicals was developed while achieving the sustainable release using chitosan nanoparticles.

The larvicidal activity of hot water, cold water, and water: acetone extracts of combined plant mixture of *Capsicum frutescens* and *Allium sativum* were tested against *Aedes* larvae and results revealed impressive larvicidal potential in cold water and water: acetone extracts. The better activity of water: acetone extract within less time period (9 hours) could be due to both water-soluble polar compounds as well as non-polar organic compounds which have been extracted into this extract compared to cold water extract. However, hot water extract did not show any significant larvae mortality, it may be due to the decomposition of active compounds with high temperature. Green *C. frutescens* fruits were used in this study because, usually it is known that mature unripe fruits have high content of bioactive compounds (such as phenolic compounds, flavonoids, ascorbic acids, etc.) compared to ripened fruits (Benchikh, et al., 2014).

According to prior literature, individual extracts from both plant materials have been documented for larvicidal properties (Kala, et al., 2010; Vasconcelos, et al., 2014; Muturi, et al., 2018; Kasim, et al., 2019). Nevertheless, our study reveals larvicidal activities that are

slightly higher when compared to the findings reported in earlier studies. This discrepancy may be attributed to geographical variation in the collection of plant materials and differences in the extraction methods employed. Furthermore, it is essential to consider that the mosquito populations subjected to testing in our study differ, and potentially more susceptible to the active components present in the plant materials compared to the larval population used in previous investigations. Our study reports novel information about the synergistic effects of *Capsicum frutescens* and *Allium sativum*, thus no any available data for comparative analysis.

The experimental data showed water: acetone combined mixture has improved activity compared to individual plant extracts and hence synergistic activity was determined by calculating Combination Index (CI index). Calculated combination index was less than one (0.001 and 0.002 for 24 and 48 exposure periods respectively) and that indicates the water: acetone extract of mixture of green *C. frutescens* and *A. sativum* showed synergistic larvicidal properties and hence the combined mixture of green *C. frutescens* and *A. sativum* was used for the encapsulation process. Even though, there are no studies on the combined effect of the subjected plants in this study, few studies were reported on larvicidal activity of combined plant extracts against mosquito larvae (Harve & Kamath, 2004; Hari & Mathew, 2018; Rajasekaran & Duraikannan, 2012; Grzybowski, et al., 2013). As examples, Grzybowski et at (2013) reported the synergistic larvicidal effect of ethanolic extracts of *Annona muricata* seeds and *Piper nigram* against *Aedes aegypti*. Furthermore, Harve and Kamath (2004) have reported the synergistic larvicidal activity of acetone and petroleum ether extracts of *Murraya koenigii, Coriandrum sativum*, *ferula asafoetida*, and *Trigonella foenum*. Those studies have shown the presence of relatively strong larvicidal activities of combined plant extracts compared to individual plant extracts. Similarly, the results of our study revealed the strong larvicidal effect of combined plant extracts. However, the lethal concentrations of our study are not able to compare with the available literature due to the nature of the extractions and the used plant materials as this is the first study which reports the larvicidal activity of combined plant mixture of *C. frutescens* and *A. sativum.* Prior studies showed that *Capsicum frutescens* are rich in phytochemicals such as alkaloids, tannins, steroids, glycosides, saponins and phenols (Anand, et al., 2018; Vasconcelos, et al., 2014) as well as *Allium sativum* contains bioactive compounds such as organosulfur, phenols, flavonoids, alkaloids, amides and saponins (Kala, et al., 2010; Muturi, et al., 2018). The combination of these two plant materials contains high content of phytochemicals which may be responsible for larvicidal activity.

One of the major concerns of the natural larvicidal material is the shorter persistence in water as it would be affected by various bioenvironmental factors such as temperature, pH, and sunlight. Hence, the frequent application of the larvicide is needed. In order to address these issues, plant material encapsulated water insoluble nano matrix was developed. Chitosan nanoparticles are prepared by ionic gelation method which based on ionic crosslinking between positively charged amino groups of chitosan and negatively charged groups of sodium tripolyphosphate (TPP) by formation of electrostatic interactions (Kashyap, et al., 2015). Tween 80 is used as a surfactant that stabilizes the formulation and avoids agglomeration (Valle, et al., 2020). We maintained the optimized conditions to obtain the minimum particle size with high porosity. The pH of the chitosan solution and TPP solution were maintained at 4.8 and 5.2 respectively to keep the optimal particle size. In higher pH, partial deprotonation of NH_3^+ in chitosan could result lower concentration of NH_3^+ to exposure to crosslinking with TPP resulting in bigger particles. Further in high pH, as the concentration of OH^- in TPP

solution is high, it could compete with PO_4^{3-} present in TPP to diffuse into chitosan resulting in large particle distribution (Mattu, et al., 2013).

Larvae mortalities were calculated for the plant extract encapsulated chitosan nanoparticles after 24- and 48-hours exposure period and empty chitosan nanoparticles (without plant extracts) were used as the control. Here, chitosan nanoparticles with and without plant extracts were placed in sachet packets and dipped in a water solution that contains *Aedes* larvae. Anand et al (2018) have reported that chitosan has a larvicidal activity without any explained mechanism of action. In contrast, our results showed that chitosan does not exhibit any larvicidal activity, as indicated by 0% mortality in the larvae for the control experiment. One possible reason for the lack of effect of chitosan nanoparticles is that the use of semi permeable sachet packets, which prevent the direct contact of chitosan and larvae.

There are reported data on larvicidal activity of essential oil encapsulated chitosan nanoparticles against mosquito larvae (Ferrerira, et al., 2019; Gonzales, et al., 2017; Zarenezhad, et al., 2021). As an example, Ferrerira et al (2019) investigated the larvicidal activity of *Siparuna guianensis* essential oil encapsulated chitosan nanoparticles against *Aedes* larvae and similar results showed with our study as 100% larvae mortality up to seven days. However, the encapsulation efficiency of our study is high (96.10%) compared to Ferrerira et al (2019). In addition, Gonzalez et al (2017) have synthesized two different essential oil encapsulated nanoparticles using polyethylene glycol and chitosan against *Culex* mosquito larvae. The encapsulation efficiency of their study is also relatively low. In addition, the time period that shows 100% larvicidal activity is also less compared with our study. As far as we understand, no earlier studies have been done on the larvicidal activity of combined plant extracts of *C. frutescens* and *A. sativum* or the combined extract encapsulated chitosan nanoparticles as well as it showed higher encapsulation efficiency and higher time period that last the larvicidal activity.

It is essential to determine the shelf life of any plant product both to recommend it as a marketable product and to report the required storage conditions (Richards et al., 2014). To evaluate the long-lasting larvicidal properties, sachet packets containing chitosan nanoparticles encapsulated with plant extract were tested over a 14-day period. The results indicated robust activity of the sachet packets up to day 10. The decline in efficiency observed after day 10 may be attributed to the degradation of the bioactivity of the compounds. Similar findings were reported by Ferreira et al. (2019), where strong larvicidal activity of their products persisted for a week. The 50% reduction in activity after seven days was sustained for nearly 19 days. No studies were found that employed sachet packets to comparatively assess our observations. Furthermore, there are no reports in the literature regarding the use of semi-permeable sachet packets to deliver plant extract encapsulated in chitosan nanoparticles for applications. The shelf life of the plant extract encapsulated nanoparticles, tested under three different temperatures, revealed that the estimated shelf life of this product is more than 6 months. No prior reported data on the shelf life study of plant extract encapsulated chitosan nanoparticles as a mosquito larvicide is available.

Plant products demonstrate greater effectiveness compared to commonly used chemical larvicidal agents such as organophosphates (e.g., temephos, fenthion, and pyrethroids) for mosquito larvae control (Harve & Kamath, 2004; Pilaquinga et al., 2019). The extensive use of chemical larvicides has led to the development of resistance in the targeted vectors. In a study by Harve and Kamath (2004), the larvicidal activity of temephos and fenthion, as well as a combination of plant mixtures, was investigated. Additionally, they explored the residual effects of both natural and synthetic larvicides. The results indicated that both natural and synthetic larvicides demonstrated high mortality rates against mosquito larvae. However,

chemically synthesized larvicides exhibited a higher residual effect compared to natural larvicides. Consequently, the efficacy of synthetic chemical insecticides was found to be lower. While high concentrations of chemical larvicides may lead to residual effects, natural larvicidal agents, due to their degradation over time in the environment, may not exhibit the same prolonged residual effect. According to the WHO standard for *Ae. aegypti* larvae, the LD_{50} values for the temephos insecticide range from 0.003 mg/L to 0.025 mg/L. The LD_{50} values obtained from our study results are considerably higher than the reported LD_{50} of temephos for *Aedes aegypti* larvae. As we assessed the crude product of the combined plant extract (combined mixture of *C. frutescens* and *A. sativum*), direct comparison of effective concentrations required to achieve a 50% mortality rate in mosquito larval populations is not feasible. Despite the differences in units, exposure populations, and experimental setups, our study revealed significant toxicity against *Aedes* larvae. Although the LD_{50} values from our study cannot be directly compared with the available literature due to these variations, they collectively indicate strong larvicidal activity against *Aedes* larvae. This highlights the potential of plant-based larvicidal agents as more environmentally friendly and sustainable alternatives.

5. Conclusion

The present study demonstrates successful development of water insoluble natural product incorporated larvicidal agent to control dengue larvae of *Aedes aegypti* and *Aedes albopictus* using the combined plant mixture of fruits of *Capsicum frutescens* and cloves of *Allium sativum* and chitosan as the nano carrier. Water: acetone extract of matured green fruits of *Capsicum frutescens* and cloves of *Allium sativum* showed the highest larvae mortality with the lowest LD_{50} values compared to individual and other combined plant extracts while showing significant synergistic activity. Further, water: acetone extract of *C. frutescens* and *A. sativum* mixture encapsulated chitosan nanoparticles also resulted 100% larvae mortality against *Aedes* larvae and it is dose and time dependent. The encapsulation efficiency is 96.10 ± 2.77% and the releasing efficiency is 90%. Encapsulated plant extract was released to the medium up to 14 days. However, the larvicidal activity of one sachet packet with 6 g of encapsulated product was 100% only for 10 days. The shelf-life of the encapsulated product was also investigated and found that it could be stored for six months without loss of the product activity. These empirical results reveal that this product could be an excellent eco-friendly natural larvicidal agent against dengue mosquitoes.

Statements and Declaration

The authors have no conflicts of interest to declare.

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