Neem oil-loaded cross-linked biodegradable polymeric capsules: Its larvicidal activity against *Culex quinquefasciatus* larvae

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**Abstract**

**Background:** Pesticide delivery system has been formulated in the form of emulsifiable concentrates, water solutions, aerosols, or spray formulations. However, such formulations showed health hazards. Encapsulation technique is the more suitable method to reduce health hazard and to deliver and release the pesticides. Natural biopolymers have been widely studied for encapsulation of pesticide compounds, as they are biodegradable, biocompatible, and low toxic to mammalian. Neem oil has been reported for controlling of the mosquitoes and more eco-friendly insecticide than synthetic insecticides. The present study was designed to prepare a cross-linked polymeric network capsules loaded with neem oil as effective controlled release formulation against *Culex quinquefasciatus* larvae.

**Materials and Methods:** Neem oil-loaded chitosan/alginate/gelatin capsules were prepared by cross-linking method. Neem oil-loaded capsules were characterized with respect to their capsule size, scanning electron microscopy (SEM) analysis, and swelling property. *In vitro* larvicidal activity of neem oil-loaded polymeric capsules was studied against *C. quinquefasciatus* larvae.

**Results:** The cross-linking method produced spherical shape of neem oil-loaded capsules. Ultraviolet spectroscopy analysis indicated that 10% of neem oil was loaded with capsule. A swelling study indicated that swelling of the loaded capsules tends to be more stable. SEM analysis showed that loading of the neem oil with the capsules fills all pores and capsules were found with good compatibility between chitosan, alginate, and gelatin due to the uniform shape of the capsule. Formulated neem oil-loaded capsules showed potential larvicidal activity (100% of mortality) against *C. quinquefasciatus* larvae in an *in vitro* model.

**Conclusion:** Formulated neem oil-loaded capsules showed a simple method of preparation and eco-friendly. These polymeric capsule containing neem oil exhibited potential larvicidal activity against *C. quinquefasciatus* larvae.

**Keywords:** Capsules, *Culex quinquefasciatus*, larvicidal activity, neem oil

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INTRODUCTION

Mosquitoes cause a major public health menace as vectors of serious human diseases. Mosquitoes belonging to the genera *Anopheles, Culex,* and *Aedes* are the vectors responsible for the transmission of different diseases such as malaria, filariasis, Japanese encephalitis, dengue and dengue hemorrhagic fever, epidemic polyarthritis, yellow fever, and chikungunya. *Culex quinquefasciatus* is the principal vector of lymphatic filariasis (Bernhard *et al.*, 2003). Filariasis is an endemic, disabling, and disfiguring disease.¹

Different approaches have been developed to control mosquito menace. One such approach to prevent mosquito-borne disease is by killing mosquito at larval stage. The current approach for mosquito control is based on synthetic insecticides. Even though they are effective, they resulted in many problems such as insecticide resistance, pollution, and toxic side effect on human begins. One of the most effective alternative approaches under the biological control program is to explore the floral biodiversity and enter the field of using safer insecticides of biological origin as a simple and sustainable method of mosquito control.²³

Larvicides are agents that kill insect larvae. Larviciding can reduce overall pesticide usage in a control program by reducing or eliminating the need for ground or aerial application of chemicals to kill adult mosquitoes. Neem plant belongs to the genus *Azadirachta* of which (*Azadirachta indica*) native of India, belonging to the Family Meliaceae.⁴ Azadirachtin is a biologically active compound in *A. indica* which has been promoted as a new insecticide that is considered more eco-friendly insecticide than synthetic insecticides.⁴

Development of controlled release formulations with environmentally friendly application depends on using of natural biodegradable materials instead of synthetic polymers. Natural biopolymers such as chitosan and alginate used for encapsulation are biodegradable, biocompatible, and low toxic to mammalian cells.⁶

Aim

The present study aims to prepare and develop a cross-linked polymeric network capsule-loaded neem oil and investigation of its larvicidal activity against *C. quinquefasciatus*.

MATERIALS AND METHODS

Materials

Neem oil was purchased from local Ayurveda market of Thrissur, Kerala. Biopolymers such as chitosan, sodium alginate, and gelatin were purchased from Sigma Aldrich, Mumbai.

Mosquito species

*C. quinquefasciatus* larvae were obtained from susceptible reared strain provided by the National Institute for Communicable Diseases, southern India branch field station located in Mettupalayam (Coimbatore district, Tamil Nadu, India), and reared under laboratory conditions. Approximately, 400–600 *C. quinquefasciatus* larvae were reared in white enameled and shallow trays containing 2–3 L of dechlorinated water. The room is maintained at temperature approximately 28°C ± 2°C and relative humidity 70%–90%.

Preparation of neem oil-loaded chitosan/alginate/ gelatin capsules by cross-linking method

Chitosan/alginate/gelatin capsules were prepared by cross-linking with glutaraldehyde. Sodium alginate solution 1% (w/v) was prepared in distilled water and stirred mechanically until dissolved completely. About 2.5% (w/v) of gelatin solution was prepared in distilled water. Chitosan solution 1% (w/v) was prepared by dissolving chitosan in 1% acetic acid solution. For the capsule formation, 25 ml of 1% (w/v) sodium alginate solution containing 2 ml of glutaraldehyde solution was placed in a beaker and another beaker containing a mixture of 12.5 ml of 1% (w/v) chitosan solution and 12.5 ml of 2.5% (w/v) gelatin solution. Chitosan/gelatin solution was then dropped by a syringe into a sodium alginate/glutaraldehyde solution contained in a beaker at 25°C. After the dropwise addition, product with spherical shape was obtained. The capsules were dried and kept in desiccator until further use. Capsules with neem oil were prepared by the same method by mixing neem oil (1 g) with chitosan solution.

Characterization of neem oil-loaded chitosan/alginate/ gelatin capsules

Measurement of capsule size

Dried form of prepared capsules was selected and their sizes were measured using digital Vernier caliper.

Scanning electron microscopy analysis

The main purpose of scanning electron microscopy (SEM) analysis is to observe the surface morphology of the prepared capsules. The instrument used was JEOL JSM-5300 microscope. The dry gel spheres were mounted on metal stubs with double-sided tape, sputtered with gold, and viewed in the SEM. The observations were performed at an accelerating voltage of 15 Kv.
Swelling properties
The swelling studies of the loaded and unloaded capsules were demonstrated by the following method. The water uptake ability of capsules was determined by immersing a definite weight of dried capsules into distilled water and allowed to swell and then by recording the water gain at different time periods up to 10 days. Excess surface water was removed by being wiped with soft paper, and the percentage of swelling was calculated by the following equation:

\[ \text{Swelling (\%)} = \frac{(W_t - W_0)}{W_0} \times 100 \]

Where, \( W_t \) and \( W_0 \) are the weights of swollen sample at time \( t \) and that of the original sample, respectively. Experiments were conducted in triplicate, and the obtained data were averaged.

In vitro larvicidal activity against Culex quinquefasciatus larvae
Larvicidal activity of neem oil-loaded chitosan/alginate/gelatin capsules was assessed according to the WHO standard procedure with slight modifications. The first instar larvae of \( C. \) quinquefasciatus were exposed to different weight of neem oil-loaded capsules. About 100 ml of running water was taken in series of 250-ml glass beakers. Four different weights of capsules (5, 10, 20, and 50 mg) were immersed into 100 ml of running water contained 50 instars of \( C. \) quinquefasciatus larvae in the beaker, and larvae death was monitored as a function of time daily for a period of time without change of water but with fresh larvae. Control sample was maintained by keeping larvae in the running water under the same conditions with unloaded capsules. The number of dead larvae at the end of 24 h was recorded, and the mortality percentage values were calculated. This experiment was repeated three times. The percentage mortality was calculated using the following equation:

\[ \text{Percentage of mortality} = \frac{\text{Number of dead larvae}}{\text{number of larvae introduced}} \times 100 \]

Statistical analysis
All the obtained data were analyzed using SPSS statistical software version 13.0 software program (Oxzus Solutions, Cochin, Kerala, India). Data of average larval mortality were subjected to probit analysis for calculating \( LC_{50} \) and \( LC_{90} \) values which were assessed by fitting a probit regression model.

RESULTS AND DISCUSSION
Formulation of neem oil-loaded chitosan/alginate/gelatin capsules by cross-linking method
Neem oil-loaded chitosan/alginate/gelatin capsules were prepared by cross-linking method. In the present study, the reaction between chitosan (1%) and sodium alginate (1%) cross-linked with glutaraldehyde (2%), and their combination with gelatin (2.5%) produced spherical capsules. The capsules were in yellow color before drying and brown in color after drying. The same method was followed to prepare the loaded capsules. Neem oil was used to prepare the loaded capsules. Capsules loaded with neem oil, as determined by ultraviolet (UV) spectroscopy at 270 nm. Chitosan and alginate react via ionic interaction between the carboxyl residues of alginate and the amino terminals of chitosan. The interaction could also be via intermolecular hydrogen bonding and the reaction mechanism between chitosan and alginate in cross-linking. This complex reduces the porosity of the alginate capsules and decreases the leakage of the encapsulated substances. Our study indicated that unloaded capsules are bigger in diameter (985 \( \mu \)m) than that loaded with the neem oil (916 \( \mu \)m) which gave 800 and 2350 capsules/g, respectively [Table 1]. Capsules were loaded with 10% of neem oil, as determined by UV spectroscopy at 270 nm.

Characterization of neem oil-loaded chitosan/alginate/gelatin capsules
Scanning electron microscopy analysis
SEM study indicated that unloaded capsules were characterized irregular in shape with cavities and yellow gold in color [Figure 1] and their surfaces morphology were examined under power of magnification \( \times 5000 \) and appeared a lot of pores and wrinkles. After loading with the neem oil, the capsules appeared under SEM as oval in shape with cavity and its surface morphology under power of magnification \( \times 5000 \) seemed more rough with scars. This confirmed that the loading of the neem oil on the capsules fills all pores. It was found that the capsules were characterized oval in shape, which shows a good compatibility between chitosan, alginate, and gelatin.

![Figure 1:](image-url)

Figure 1: (a) Neem oil-loaded chitosan/alginate/gelatin capsules; (b) scanning electron microscopy analysis of unloaded chitosan/alginate/gelatin capsules; (c) scanning electron microscopy analysis of neem oil-loaded chitosan/alginate/gelatin capsules.
biopolymers. The capsules diameter was found to be 900–1000 μm in size.

**Swelling properties**

The swelling ability of the capsules is an important factor to evaluate its property, used as scaffold materials for controlled neem oil release in water to play an active role as a larvicide for a long period. Swelling property refers to the hydrophilic groups and more positively charged amino groups along of the biopolymer chains lead to much-expanding polymer network. In addition, the swelling behavior of the capsules mainly results from the electrostatic repulsion between the negatively charged carboxyl groups on alginate and gelatin.[10] In our study, swelling studies of unloaded and loaded capsules with neem oil has been shown in Figure 2. Swelling capacity of loaded and unloaded capsules was studied for 10 days of the experiment. The unloaded capsules gradually increased in their weight and exhibited a higher water absorption rate than the loaded capsules through the first 6 days.

![Swelling studies of unloaded capsule without neem oil and loaded capsules with neem oil](image)

**Table 1: Properties of chitosan/alginate/gelatin capsules unloaded and loaded with neem oil**

| Parameters                          | Unloaded capsules | Capsules loaded with neem oil |
|-------------------------------------|-------------------|-------------------------------|
| Components ratio (ch:alg:glut:neem oil (%)) | 1:1:2:5:2:0       | 1:1:2:5:2:1                   |
| Capsule color                       | Yellowish-brown   | Yellow                        |
| Weight before drying                | 18.24             | 11.46                         |
| Weight after drying                 | 2.43              | 1.02                          |
| Capsule (no/g)                      | 800               | 2350                          |
| Flexibility                         | Flexible          | Flexible                      |
| Before drying                       | Hard              | Hard                          |
| After drying                        |                   |                               |

The capsules rapidly swelled within the first 24 h. The highest swelling degree was obtained at the 3rd day (260%); however, it was decreased to 50% on the 7th day and then remained constant up to the 10th day. However, it can be noted that the swelling of the loaded capsules tends to be more stable along the experiment (15%–50%). This result may be due to the reduction in the porosity of capsules thereby decreases the sorption of the water into capsules.

**In vitro larvicidal activity against Culex quinquefasciatus larvae**

Neem oil-loaded chitosan/alginate/gelatin capsules were subjected to larvicidal bioassay using running water as vehicle. A total number of 50 larvae were exposed to different weight of the neem oil-loaded biodegradable capsules. The observed percentage of mortalities of *C. quinquefasciatus* larvae based on probit regression analysis for different weight of the neem oil-loaded biodegradable capsules was shown in Table 2. Awareness has been considered to use natural, eco-friendly pesticide compounds for larvicidal activity. At present, chemical pesticides are not safe for controlling of mosquito due to pesticide resistance by vectors as well as environmental imbalance. Further, continuous usage of chemical pesticides can produce harmful effects, and chemical pesticides do not give absolute results for controlling of mosquito. Therefore, alternative methods for mosquito control are needed.[11] Other scientific studies on larvicidal activity, Bhan *et al.*, 2014, showed that the highest mortality rate was observed by the slow release of lesser amount of pesticides after encapsulation: 0.003 mg/L for 8% temephos formulation and 0.019 mg/L for 4% imidacloprid as compared to their noncapsulated form of temephos and imidacloprid (0.004 and 0.021 mg/L) after 72 h of exposure against *C. quinquefasciatus* larvae. Based on this study, encapsulated form of pesticides is considered economical and eco-friendly due to controlled slow release of pesticides.[12] Tsuji, 2001, reported that development of highly effective and reduced dose of pesticides is needed. Micro-encapsulation techniques could be suitable for improved handling safety of pesticides with exposure and hazard reduction as well as masking of odor of pesticides.[13] A study of

**Table 2: Observed percentage of mortality of Culex quinquefasciatus larvae exposure to neem oil-loaded chitosan/alginate/gelatin biodegradable polymeric capsules**

| Weight of the capsule with neem oil in 100 ml of running water | Number of larvae exposed | Number of larvae dead | Percentage of mortality | Probit for larvicidal | LD₅₀ | LD₉₀ |
|---------------------------------------------------------------|--------------------------|----------------------|------------------------|-----------------------|------|------|
| 5 mg of capsule                                               | 50                       | 14                   | 28                     | 4.42                  | 16.32| 44.10|
| 10 mg of capsule                                              | 50                       | 22                   | 44                     | 4.85                  |      |      |
| 25 mg of capsule                                              | 50                       | 34                   | 68                     | 5.47                  |      |      |
| 50 mg of capsule                                              | 50                       | 48                   | 96                     | 6.75                  |      |      |

Mortality rate was analyzed using probit regression analysis
et al., 2014, indicated that encapsulation of hydrophilic methomyl in shell cross-linked nanocapsules formed by the self-assembly of photo-cross-linkable carboxymethyl chitosan followed by the rapid UV irradiation was attained. The shell cross-linking and its degree of cross-linking were responsible for the diffusion behavior of pesticide.\textsuperscript{14}

In our study, results indicated that larvicidal activity of neem oil-loaded biodegradable capsules against \textit{C. quinquefasciatus} larvae was gradually increased based on the weight of the neem oil-loaded biodegradable capsules [Figure 3]. The LC\textsubscript{50} and LC\textsubscript{90} values (95% confidence intervals) for neem oil-loaded biodegradable capsules were found to be 16.32 and 44.10, respectively. With this information, we found that neem oil-loaded biodegradable polymeric capsules have a potential larvicidal activity against \textit{C. quinquefasciatus}.

**CONCLUSION**

On the basis of the above results, biodegradable capsules with the combination of chitosan/alginate/gelatin were effectively formulated to encapsulate the neem oil for the larvicidal activity against larvae of \textit{C. quinquefasciatus}. The synthetic or chemical pesticides can be available easily with low cost. However, the use of the natural medicinal oils for controlling of mosquito larvae offers a safer product, easy to handle, and safer alternative. The biodegradable polymeric capsules are eco-friendly materials and could be suitable to encapsulate the neem oil for larvicidal activity. Therefore, this study concluded that the encapsulated of neem oil with biodegradable polymeric capsules showed potential larvicidal activity against \textit{C. quinquefasciatus} larvae.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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