

EFFICIENCY STUDY OF NEEM SEEDS-BASED NANOBIOPESTICIDES

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EFFICIENCY STUDY OF NEEM SEEDS-BASED NANOBIOPESTICIDES

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Abstract. An innovative nanosized natural pesticide based on neem seeds was developed. The resulting nanobiopesticide (NBP) was synthesised by entangling neem seed extract on chitosan cross-linked with succinic anhydride *via* ultrasonic treatment following purification. Fourier-transform infrared (FTIR), ultraviolet-visible (UV-Vis) and dynamic light scattering (DLS) spectrophotometers were used to characterise the resulting NBP, and its stability was observed against changes in pH, temperature and UV radiation.

Keywords: nanobiopesticide, neem seed extract, chitosan, succinic anhydride.

1. Introduction

In Indonesia, the agriculture sector plays an important role in the overall national economy, and it is the sector that contributes the most to the labour force. According to the Statistical Bureau, in 2013 the agricultural sector hired up to 40 million people from a workforce of 114 million [1]. Crop failure is the thing most feared by farmers, and it is usually caused by pests that attack crops. The use of synthetic pesticides is regarded as the best solution for overcoming this problem, although, in reality, it has many negative impacts on the environment and the animals who reside in and around agricultural land. An alternative solution is the use of natural pesticides or biopesticides, such as neem seeds, even though some modification is needed to improve the performance and quality of these types of pesticides. Nanoencapsulation is a new, attractive technology that has been extensively developed; it covers active pesticides with a polymer coating to produce nanosized particles.

The most widely used polymer layer is chitosan. Chitosan is a biodegradable [2], bio-adhesive material [3]. Under specific conditions [4], it forms a gel and it will expand the polymer solubility and control the release of active substances, which cover most of the pores [5, 6].

The development of nanotechnology has benefited a wide range of sectors, including health [7, 8], electronics [9], sustainable energy [10], the environment [11], and many others. In the agriculture sector, nanotechnology has had a significant impact in terms of enhancing the agricultural process [12]. The present study focused on developing a nanoencapsulated extract of neem seeds with chitosan modified (cross-linked) by succinic anhydride using ultrasonication. The resulting nanobiopesticide (NBP) was determined to be an improvement over synthetic pesticides by studying its stability against various pH and temperature ranges, and under UV radiation along with the controlled release study of the active ingredient. The resulting product was characterised using Fourier-transform infrared (FTIR), ultraviolet-visible (UV-Vis) and dynamic light scattering (DLS) spectrophotometers to determine the success of the generating process. The efficiency of the encapsulation process was accessed by investigating the toxicity of the prepared NBP against *Spodoptera litura* (*S. litura*).

2. Experimental

2.1. Synthesis of Chitosan Modified with Succinic Anhydride

About two grams of chitosan is dissolved in 40 ml of 10% acetic acid and 160 ml of methanol. On the other part, about one gram of succinic anhydride is dissolved in 30 ml of acetone. Both solutions are subsequently mixed simultaneously and then stirred for 24 h until a gel is formed, following with deionized water (DI) addition as much as 200 ml when the mixture has successfully formed the gel. The next process is the conditioning mixture at pH 10 with the addition of NaOH dropwise. The next

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process is dialysis of resulted crosslinked chitosan using 50 kDa MWCO membrane for 24 h agitation.

2.2. Synthesis of NBP

The study followed the encapsulation process used by Fahmi *et al.* [13], with some modifications, to meet the desired goals. Experimentally, about 10 ml of chitosan modified with succinic anhydride were mixed with 0.5 ml of neem seed extract dissolved in *n*-hexane. Mixing these two solutions produced two phases. The mixture was then ultrasonicated for 2 min with a power of 5 % (25 Hz) at room temperature to obtain a homogeneous solution and to let the neem seed extract be encapsulated with the chitosan modified with succinic anhydride. During the ultrasonication process, the encapsulated extract that was successfully covered by chitosan will move to the water phase. The results obtained from the water phase were further analysed using 50 kDa molecular weight cut-off (MWCO) membrane in DI water, and stirred for 24 h. The resulting mixture was called NBP.

2.3. Rearing Armyworms in a Laboratory

Larvae of *S. litura* were collected from a brassica field in Batu, East Java. Then, the larvae were reared in plastic jars with brassica leaves as feed. The hatch eggs developed into stage 1 larvae, stage 2 larvae, *etc.* At stage 3, the larvae were used in an insecticidal bioassay.

2.4. Neem Seed Extraction

The neem seeds were initially oven-dried at 333 K until a constant weight was obtained; then they were finely grounded. Ethanol was added to the powder and the mixture was stirred for 2 days until it became uniform. It was then subjected to filtration. The obtained solid mass was washed with chloroform, which was then evaporated. The resulting powder was the neem seed extract.

2.5. Encapsulation of the Neem Seed Extract

The neem seed extract was dissolved in hexane and the chitosan was dissolved in acetic acid solution. After these two solutions were mixed, the neem seed extract was inserted into the interspace of the chitosan nanoparticle through ultrasonic wave for 2 min to homogenise the mixture. The wave treatment produces cavitation (hexane-in-water) bubbles in the medium via a series of compression and relaxation waves. The high dimension of the crosslink-chitosan results in the encapsulation of small molecules, even when present in a difference phase. The sample was cured for 24 h and then centrifuged (6000 rpm, 20 min) to separate the water

phase containing the neem seed extract. This step was followed by dialysis using a dialysis membrane (MWCO 50 kDa, 24 h).

2.6. Repellent Activity Test of the Nanoencapsulated Neem seed extract towards Armyworms (*S. litura*)

The larvae that were of the same age as a result of the rearing were used to conduct the repellent activity test. Complete randomized design (CRD) was used for the treatment of nanoencapsulated neem seed extract at various concentrations: 0 % (using only aquadist), 10 %, 20 % and 30 % (v/v). Each treatment was repeated six times for total of 24 treatments. The experiment was conducted in the laboratory using plastic jars that were covered with gauze. In every treatment unit, 20 tested larvae were infested in the jars and *Brassica chinensis* var. *parachinensis* leaves were used as a feed. The observed parameter is the percentage of tested larvae that stayed away from the feed due to the nanoencapsulated neem seed extract treatment. After the treatment was applied, the samples were observed for seven days. The repellent activity percentage (% RA) was adjusted using the following equation:

$$\% RA = \frac{LF}{LT} \cdot 100\%$$

where LF refers to the number of larvae which stay away from feed and LT refers to the total number of tested larvae.

2.7. Data Analysis

The collected data were statistically analysed by using ANOVA (Analysis of Variance). Comparison of treatment means was performed using LSD (Least Significant Difference) test at $p < 0.05$ probability level.

2.8. Characterizations

Zetasizer ZS 90 (Malvern, USA) was used to perform the size distribution and zeta potential value of resulted NBP. The concentration of neem seed extract adjusted by calibration curve based on light adsorption measured by UV spectrophotometer UV-1800 (Shimadzu, Japan). The standard solution of neem seed extract was initially a concentrated blackish brown diluted one in ethanol at a concentration of $6 \cdot 10^{-2}$; $8 \cdot 10^{-2}$; $10 \cdot 10^{-2}$; $12 \cdot 10^{-2}$ and $14 \cdot 10^{-2}$ M. The maximum wavelength of NBP can be observed by UV-Vis spectrophotometer, where a maximum wavelength of neem seed extract is around 276 nm. The maximum wavelength that has been obtained can be used to find the concentration of the standard solution through the absorbance value. Vibration spectra of a sample are performed using FTIR spectroscopy (Shimadzu, Japan).

3. Results and Discussion

3.1. Crosslinking and Nanoencapsulation Process

The present study used the nanoencapsulation process found in [13], by modifying some parts in order to meet the desired objectives. The process of inserting the neem seed extract into the chitosan modified with succinic anhydride was done under the ultrasonic wave [14]. This wave breaks the particles into smaller sizes; it also accelerates the presence of a bubble-shaped bubble. This results in an *n*-hexane bubble containing the neem seed extract in water containing chlorine modified with succinic anhydride because the *n*-hexane contains fewer neem seed extracts than the water containing chitosan modified with succinic anhydride. The bubbles form an emulsion from the non-mixing phase. The ability of harmonic waves to suppress and attract bubbles causes the *n*-hexane bubbles in water to shrink so the wave cannot be harmonized. In this condition, there is only the neem seed extract surrounded by chitosan modified with succinic anhydride so that the neem seed extract is trapped in the anhydride-modified chitosan and driven into the water phase. Encapsulation of the neem seed extract is still at random sizes, so further treatment is needed using a membrane dialysis of MWCO 50 kDa. The 50 kDa MWCO value means that the membrane can withstand a molecule weighing 50000 Da at least 90 % or more [15]. The Zetasizer Nano ZS (Malvern Instruments) was used to determine whether the results are within the nanosize characterisation. This instrument utilises a DLS. The DLS

results showed that the particle size was 271.6 nm. Thus, it can be concluded that the encapsulation process of the neem seed extract was successfully performed.

3.2. The Stability of NBP

The stability of NBP was first observed against various pH ranges because pH is one of the important parameters of the product. This pH stability analysis was performed by adjusting the pH range from 3 to 12 (by adding HCl or NaOH solutions). The photographs (Fig. 1) show that the post-pH treatment changes were only slight, and almost no changes on its appearance and its UV absorbance at pH 3 and pH 4. Moreover, most of the changes occurred in the form of turbidity at higher pH value.

From the results of this study, it can be concluded that the nanoencapsulation product of neem seed extract can be stable at a pH range of 5–12. The results reveal that the proposed NBP will maintain its stability in the natural environment or soil that is slightly acidic to neutral (pH 5.0–7.5).

The stability of the proposed NBP under various temperatures was also assessed. The results (Fig. 2) of the nanoencapsulation neem seed extracts with a temperature treatment show that the colour or the turbidity of the extracts did not change (based on each UV maximum absorbance). It can be concluded that nanoencapsulation neem seed extracts with chitosan modified with succinic anhydride can survive in an environment with temperatures ranging from 303 to 333 K. These data prove that the NBP meets the requirements for agricultural application (average temperature 308–323K).



Fig. 1. Photos of NBP under various pH ranges (from pH 3 to pH 12). The UV absorbance maximum of each sample is showed in the lower series



Fig. 2. Photos of NBP under various temperatures: at room temperature (a), 303 K (b), 313 K(c), 323 K(d) and 333 K (e). The UV absorbance maximum of each sample is showed in the lower series

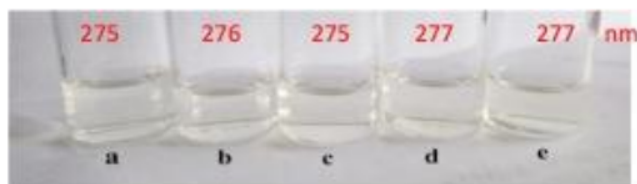


Fig. 3. Photos of NBP after UV exposure for different times (h):1 (a), 2 (b), 3 (c), 6 (d) and 12 (e). The UV absorbance maximum of each sample is showed in the upper series

Next, the stability of the proposed NBP was further assessed using UV radiation (Fig. 3). Qualitative and quantitative methods were used to analyse the stability of the nanoencapsulation neem seed extract with chitosan modified with succinic anhydride against UV radiation. The qualitatively visually observed changes are shown in Fig. 3. As seen, the NBP is stable against UV radiation because its turbidity or colour did not change. The same result was also found for the nanoencapsulation of neem seed extract without modification.

Quantitative methods are needed because qualitative testing does not give specific results. Adjusted NPB concentrations were based on the conversion results of their absorbance to calibration curve, whereas in this study, showed regression equation is $y = 0.93x - 0.0372$ with a correlation or R^2 value of 0.9951. Absorbance values obtained by irradiating a long time will be used to determine degradation reaction rate constants of the first order. It has been widely reported that most of the degradation of organic compounds has always followed the first order reaction [16].

If the absorbance and concentration values are known then we can find the k value or the rate of chemical reaction kinetics. Kinetics of chemical reactions can be expressed in an equation called the rate law that can be determined from the experimental data [17]. Value of kinetics rate commonly symbolized by " k " can be obtained from the slope between the axis (x) and the ordinate (y) which is the value of the duration of the radiation and the value of $\ln[A]$ where the value of A is the sum of concentrations after radiation. Regression values resulting from the three test materials are shown in Table 1.

The greater the value of the kinetics rate, the faster the substance will degrade. From the above data, the fastest sequence of degradation rates is the neem seed extract > nanoencapsulation of neem seed extracts without modification > nanoencapsulation of neem seed extracts with modification. Thus, neem seed extract without modifiers is less effective when used as a natural pesticide because it is easily degraded by sunlight. The similar statement is supported by a previous report, where one of the shortcomings of natural pesticides using neem seed extract is the nature that is not resistant to sunlight [18, 19].

Table 1

Degradation rate of the samples after exposure with UV light

Sample	Regression value	Kinetics rate, m/s
Neem seed extracts	$y = -0.021x + 0.749$	0.021
Nanoencapsulation of neem seed extracts without modification	$y = -0.013x - 0.207$	0.013
Nanoencapsulation of neem seed extracts with modification	$y = -0.008x - 0.282$	0.008

3.3. Release of Active Ingredients from Nanoencapsulation N eem Seed Extracts with Chitosan Modified Succinic Anhydride

The release of the NBP content was evaluated by measuring the absorbance obtained from neem seed extract released from encapsulation and successfully passing the dialysis membrane MWCO 50 kDa. The concentration of neem seed extract released at any given time can be calculated through the equation as follows [8]:

$$C_t' = C_t + \frac{v}{V} \sum_0^{t-1} C_t$$

where C_t' and C_t are the actual and apparent concentrations of neem seed extract at time t , respectively; v is the volume of the aliquots taken, ml; V is the total volume of the buffer, ml.

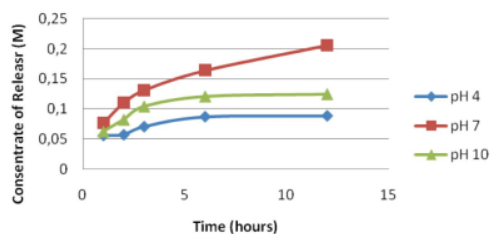


Fig. 4. Release pattern of the neem seed extract from NBP for various pH

The concentration of the extract is determined by UV adsorption of the extract comparing with a calibration curve of standard. The releases of active ingredient from nanoencapsulation neem seed extract was treated at varied values, namely at pH 4, 7 and 10.

Based on the release observations (Fig. 4), it can be seen that the maximum active substance released from NBP occurred at pH 7, followed by pH 10 and then pH 4. It is assumed that at pH 7 (neutral) the molecule is formed in the neutral charge so the interaction of the van der Waals force between the COOH group and NH₂ is weak. The weak bond increases the absorption power of the water so the index expands and causes the active substance in the nanoencapsulation to be more easily released or disengaged. In an alkaline medium (pH 10), ionization of carboxylic groups (–COOH) into a carboxylate ion (–COO[–]) occurs. This ion site has a stronger attraction to any potential electropositive group, including amino groups (NH₂), that exist on chitosan, resulting in a stronger van der Waals bond between them. This attractive force affects the release of the nanoencapsulation neem seed extract from chitosan modified with succinic anhydride. The stronger bond results in a denser nanoencapsulate, so it is difficult to release the active substances that are inside. However, with an acid medium (pH 4), the amount of dissolved chitosan with soluble succinic anhydride is relatively decreased. This is because the acidic pH range is an isoelectric point of succinic anhydride-modified chitosan, which results in the balance of NH₃⁺ and COO[–] in the molecule [20]. This condition makes it more difficult to release the active substance in the nanoscale formation due to the ion balance in the molecule. Thus, it can be concluded that NBP has a maximum discharge occurring at pH 7 (neutral). This also corresponds to the good pH criteria required for crops when released into an agricultural environment.

3.4. Characterization of NBP

FTIR was used to find out whether a chitosan was successfully modified with succinic anhydride. The result of several FTIR tests is shown in Fig. 5, with a description of the [i] line showing the extract of the neem seeds. The [ii] line is chitosan. The [iii] line indicates a modified chitosan and [iv] line indicates the nanoencapsulation neem seed extracts with chitosan modified with succinic anhydride.

Strong band of chitosan is observed at wavelength of 3600–3335 cm^{–1} referring to OH and NH functional groups. The CH stretching band appears on chitosan at 2852.81 cm^{–1} and shifts to 2920.32 cm^{–1} after the modification. Functional groups of NH primer of chitosan are clearly observed at 1639 cm^{–1}, which is slightly shifted

to 1641.48 cm^{–1} on the modified chitosan (Fig. 5a). Ester and ether functional groups formed *via* the modification process of chitosan are shown at 1151.54 cm^{–1} (Fig. 5b). When comparing several peaks above, it can be concluded that chitosan was successfully modified with succinic anhydride. Successful encapsulation process of neem seed extract is proved by existing NH band of chitosan on NPP at 1558.48 cm^{–1} (Fig. 5c) and the existence of neem seed extract band on NBP is also confirmed by the shift of C=O aliphatic esters band from 1710.92 cm^{–1} to 1633.71 cm^{–1} (Fig. 5d).

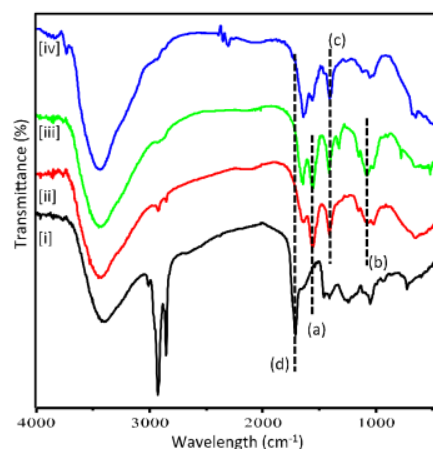


Fig. 5. FTIR pattern of neem seed extract [i], chitosan [ii], cross-linked chitosan [iii] and NBP [iv]

The characterization on using UV-Vis spectrophotometer was further done to know that the neem seed extract was successfully encapsulated by chitosan. The maximum absorbance value of neem seed extract is 276 nm, where similar one on NBP – 275 nm. Thus, it strongly reveals that neem seed extract is successfully encapsulated in the product because the maximum wavelength is almost the same.

Table 2

Size distribution and zeta potential of sample

Sample	Size, nm	Zetta potential, mV
Neem seed extract	320	35.6
Chitosan modified succinic anhydride	319	0.02
NBP before dialysis	518.8	–
NBP after dialysis	271.6	20.3

Characterization using the DLS further aims to measure the size of the particles formed and its zeta potential (Table 2). The size of neem seed extract of

320 nm and chitosan modified with succinic anhydride of 317.9 nm then showed that the extract of neem seeds was successfully encapsulated by increasing the size of 518.8 nm in the product prior to dialysis. After dialysis, the size becomes 271.6 nm. Both of these proved that the neem seed extract was successfully encapsulated in the chitosan modified with succinic anhydride and also successfully made in nanosize. Zeta potential is the parameter of electrical charge between colloidal particles. The higher zeta potential values the better is preventing the occurrence of flocculation. The potential zeta value indicates that the resulting product is relatively stable and flocculated with a relatively long time. In addition, the

potential zeta value of NBP is 20.3 mV, where there are zeta potentials of neem seed extract (35.6 mV) and succinic anhydride modified chitosan (0.02 mV). This finding also reveal the successful encapsulation process of neem seed extract.

Assessment of NBP toxicity against *Spodoptera litura* is presented in Table 3. The data showed that neem seed was quite efficient on pressing *S. litura* living. Compared with untreated leaf, mortality amount of *S. litura* significantly increases (up to 82 %). Moreover compared with bare neem seed extract, the encapsulation process enhances ability of neem seed to be an excellent pesticide, where mortality can efficiently kill *S. litura*.

Table 3

Mortality investigation of *S. litura* after treated with specific samples

Concentration of neem seed extract to total NBP, w/w	Mortality		
	Number	Percentage dead <i>S. Litura</i> from the total	Average
Control	120	0.00	0.00
Bare neem seed extract	98	81.67	16.33
0.1	117	97.50	19.50
0.2	119	99.20	19.83
0.3	120	100.0	20.00

4. Conclusions

The effort to produce nanobiopesticides from neem seed extract with chitosan modified succinic anhydride can be done through the process of encapsulation with ultrasonication. NBP performs excellent stability at pH 5–12, temperature of 303–333 K and exposure to UV radiation for 12 h. Treating the leaf with NBP can increase mortality of NBF perfectly. This finding can initiate further development of agricultural aspect based on nanotechnology.

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**ЕФЕКТИВНІСТЬ НАНОБІОПЕСТИЦИДІВ
НА ОСНОВІ НАСІННЯ НІМУ ПРОТИ
*SPODOPTERA LITURA***

Анотація. Розроблено нанорозмірний інноваційний натуральний пестицид на основі насіння німу. Новий нанобіопестицид (НБП) синтезовано нанесенням екстракту насіння німу на хітозан, зшитий янтарним ангідридом, в результаті

оброблення ультразвуком з подальшим очищенням. З використанням Фур'є-спектроскопії, УФ-спектроскопії та динамічного світлорозсіювання визначено основні характеристики НБП. Встановлено залежність стабільності НБП від зміни рН, температури і ультрафіолетового випромінювання.

Ключові слова: нанобіопестицид, екстракт насіння німу, хітозан, янтарний ангідрид.

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