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Brief Report

Toxicity nanoinsecticide based on clove essential oil against *Tribolium castaneum* (Herbst)

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This study aimed to characterize nanoparticles loaded with clove (*Syzygium aromaticum*) essential oil-based polyethylene glycol (PEG) and to know their insecticidal activity against red flour beetle (*Tribolium castaneum*). The nanoparticles have irregular shapes in good dispersion. The nanoformulation could not enhance clove oil contact toxicity to *T. castaneum*, but could protect the oil from degradation and evaporation while simultaneously allowing sustained release, as indicated by the continued high toxicity for 16 weeks of storage.

Keywords: *Syzygium aromaticum*, a botanical insecticide, nanoparticles, polyethylene glycol (PEG), sustained-release, *Tribolium castaneum*.

Introduction

Insect damage to stored grains is a major problem worldwide. About 10–40% of produced grains are lost every year due to insect damage in developing countries alone, resulting largely from the lack of modern storage technologies.[1)] Pest infestation of stored products has many disadvantages such as losses of product weight, nutritive contents, commercial and esthetic value as well as it may have a health hazard.[2)] The red flour beetle (*Tribolium castaneum*) is the most abundant and important pest of stored agricultural products.[3,4)] This insect is being mainly cereal feeders from more than 4000 years ago.[5)] It is a very common pest infesting many flour mills, warehouses, and grocery stores and it has a worldwide distribution.[6)]

The conventional way to control this insect pest has been with the use of synthetic insecticides, either directly applied to grains or by gas fumigation.[7)] Several fumigants have been tried, from the last three decades; particularly phosphine and methyl bromide had been used extensively for the disinfestation of dried fruits including dates, grains, and grain products. The Montreal Protocol has directed phasing out the use and production of methyl bromide because it is harmful to the environment and human health. It has a high potential for depleting the ozone layer because of its high chemical reactivity. Phosphine has the potential to be accepted as an alternative to methyl bromide but there are some limitations to its usage.[2)] Many of the insect populations already have high frequencies of phosphine resistant individuals.[8)] *T. castaneum* has developed resistance to chemical insecticides.[9)] There is a substantial increase in phosphine resistance in *T. castaneum* in the past 21 years, and these levels of resistance to phosphine approach those reported for other stored grain pest species in other countries. The most resistant *T. castaneum* population was 119-fold more resistant than the susceptible strain.[10)] The mortality rate of selected resistant populations, with selected susceptible ones, is inversely related to their respiration rate.[8)] However, concerns have arisen about the persistence of insecticide residues in grains which can be harmful to mammals.[7)] Therefore, finding an eco-friendly insecticide for the management of the pest is of great importance.

The phasing out of methyl bromide as a fumigant, resistance problems with phosphine and other fumigants in stored product beetles, and serious concern with human health and environmental safety have triggered the search for alternative bioinsecticide of plant origin.[11)] The presence of essential oils and their components, where natural compounds from plant sources

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are believed to have an advantage over synthetic pesticides in terms of low toxicity to mammals, rapid degradation, and local availability.[12] Some essential oils have acute toxicity, repellent action, feeding inhibition, or harmful effects on the reproductive system of insects.[7] Essential oils, steam distillation from plant leaves, and even the leaves of certain aromatic plants themselves (especially in the family Myrtaceae and Lamiaceae, and several other plant families) have traditionally been used to protect stored seeds and nuts, and to repel flying insects at home.[13] The fumigant toxicity of a large number of essential oils extracted from various spices and herb plants was assessed against several major stored-product insects. *Tribolium castaneum* (Herbst) was found to be the most resistant, compared with *Sitophilus oryzae* (L.), *Rhyzopertha dominica* (F.), and *Oryzaephilus surinamensis* (L.), to most essential oils.[1] Many aromatic plants can be used as pesticides, including clove (*Syzygium aromaticum*) with the main component of this oil being eugenol. Contact toxicity and grain treatment of eugenol against *T. castaneum* toxicity, showed eugenol was more effective on grain than on filter paper discs. Eugenol was also highly repellent to this beetle species. Development of eggs and immature stages inside grain kernels was completely inhibited by eugenol treatment.[14]

The application of essential oils to storage pest control requires an appropriate formulation formed by biodegradable compounds that protect essential oils from degradation and evaporation, while simultaneously allowing sustained release.[15] The rather significant loss of activity of eugenol within 24 hr of application reduces its protectant potential in storage.[14] It is possible to enhance its toxicity and persistence through suitable formulations such as in nanoparticle formulation. This study aimed to characterize nanoparticles loaded with clove essential oil-based polymer PEG and to know their insecticidal activity. This study can be useful in developing management strategies to control *T. castaneum*.

Materials and methods

1. Insects culture

Insects of *T. castaneum* were obtained from the collection of Plant Pest Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, University of Brawijaya, Malang. The feed used for culture is a composition of 95% white flour and 5% instant yeast.[16] Insects were identified using a stereomicroscope to determine male and female insects. The feed is put into a glass jar and mixed evenly, then carried out 100 imago infestations with a ratio of male and female 1 : 4 into the feed. Furthermore, the culture jar is allowed for seven days for the oviposition process. Then, all the adults are removed from the culture jar. Insects are maintained in laboratory conditions (temperature 27±2°C and relative humidity of 70±5%) and without insecticide treatment for several generations. Adult insects emerging, aged 1–14 days, used in this study.

2. Preparation of clove oil nanoparticles

The main ingredients in the preparation of this nanoparticle are clove essential oil obtained from a commercial company and polyethylene glycol (PEG) 6000 from Merck (Hohenbrunn, Germany). The nanoparticles are prepared by melting dispersion method with some modifications.[17] Several parts of PEG 6000 (100 g per part) were heated at 65°C and use a magnetic stirrer thermostat. After melting 10.0 g of clove oil separately each mixed with PEG. To ensure the distribution of clove oil in the PEG, the mixture is stirred strongly for 30 min. Then, the mixture was cooled to 12°C for 12 hr to immediately form the nanoparticles; all were grinded in the cold mortar at 12°C and sieved using a 230 mesh sieve. The powder is placed in an airtight polyethylene bag and stored at 27±2°C in a desiccator containing calcium chloride to prevent water absorption before further testing.

3. Characterization of clove oil nanoparticles

3.1. Visualization of clove oil nanoparticles

Nanoparticles with a 10% ratio of clove oil for PEG was used in this test. Some of the nanoparticles dissolved in an absolute ether for 10 min, for which they were used sonication, to form a homogeneous solution. A drop of homogeneous solution was transferred to a carbon-coated copper grid, followed by negative staining with a phosphotungstate acid solution (2%, pH=6.7) for 1 min. After that was dried at room temperature (25°C), the image was visualized with a transmission electron microscope (TEM) at 80 kV.

3.2. The encapsulation efficiency of clove oil in nanoparticles

Nanoparticles of clove oil have been mixed with absolute ethanol–H_2O (75 : 25) and heated for 30 min at 50°C. For each mixture, a dilution series is made to obtain different concentrations. A UV-visible spectrophotometer (Shimadzu, Japan) was used to colorimetric tests for the absorbance of each concentration. From the obtained concentration plot calibration to the absorption of nanoparticles from clove oil-PEG. After storage of 5 day, several parts of nanoparticles (0.1 g per part) were dissolved in 2 mL of absolute ethanol–H_2O (75 : 25) and heated for 30 min at 50°C, to dissolve completely. The solution absorbance is set at 285.5 nm, and the values were compared with the standard curve. The efficiency of the encapsulation of clove oil is calculated by comparing these observations with the original amount of inserted clove oil. Every test is repeated 3 times.

3.3. Size and distribution of clove oil nanoparticles

A dynamic light scattering (DLS) particle size analyzer (Malvern Instruments Zetasizer Nano-S) was used to determine the average size and distribution. After 3 days of storage 0.2 g of samples of clove nanoparticles at 10 mL of distilled water were suspended. The suspension then was filtered using filter paper from Wathman No. 1. Every test is repeated 3 times.

3.4. Clove oil composition

Gas Chromatography-Mass Spectrometer (GC-MS) (Shimadzu, Japan) was used to determine the composition of the clove oil in pre/post-nano formulations. For the extraction of the oil, the sample of 0.5 g was dissolved in 5 mL of distilled water and then heated for 30 min at 50°C; then used to obtain the extracted clove oil 4 mL of absolute ether. Mass Spectrometer condition: helium carrier gas with a flow of 1.34 mL/min); injector temperature of 250°C; interface temperature of 240°C; source temperature of 200°C; column temperature programmed at 60°C with a rise of 10°C/min to 230°C; mass spectrum carried out at 70 eV. Compounds were classified by comparing their retention times with known compounds and comparing their mass distribution with those contained in the Wiley 8 Library (comparative content >80%). The relative percentage number is directly derived from the GC peak region.

4. Residual contact toxicity

Clove oil nanoparticles were used to treat samples of 20 g rice. The concentrations of clove oil ranged from 0.9 to 1.2% and of clove oil nanoparticles ranged from 12.6 to 15.2% (w/w) (same concentration used to calculate the efficiency of clove oil encapsulation). For oil, the rice samples were treated with 3 mL of the hexane clove oil solution and allowed to dry for 2 hr, before being placed and sealed in 100 mL glass containers. Clove oil nanoparticles were combined with rice and shaken vigorously to spread the compound. Samples of rice treated with hexane or PEG 6000 alone have been used as controls. The research was performed on a routine basis until 16 weeks. As many as 20 insects are introduced for each time.

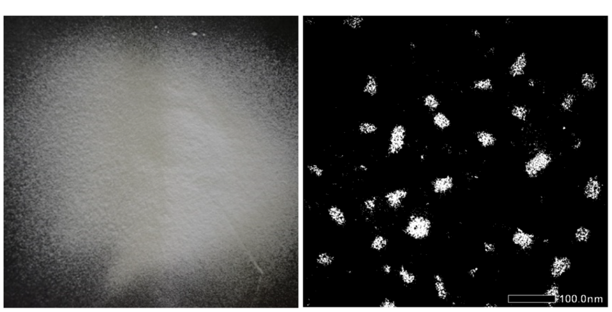
5. Statistical analysis

The Abbot (1925) based estimate of the percentage of corrected mortality. To get the LC50 value, the mortality data were subjected to probit using the statistical program SPSS 13.0.

Results

1. Morphology of clove oil nanoparticles

The clove oil nanoparticles produced are in the form of dry solid powder (Fig. 1A). The morphology of nanoparticles at the 10% ratio of clove essential oil to PEG was visualized by transmission electron microscope (TEM). The obtained nanoparticles appearing has irregular shapes in good dispersion (Fig. 1B).



A B

Fig. 1. Images of polyethylene glycol (PEG) coating nanoparticles loaded with clove oil. A. Powder image of nanoparticles, B. Transmission electron microscope (TEM) image of nanoparticles.

Table 1. The Z-average size, polydispersion index (PDI), and encapsulation efficiency of the clove oil nanoparticles at 10% ratio clove oil-PEG.

Parameter	Value±S.E.
Size (nm)	179±1.69
PDI	0.24±0.007
Encapsulation efficiency (%)	77±1.3

* SE=Standard error

2. Size, polidispersion index (PDI), and encapsulation efficiency of clove oil nanoparticles

The results of size, polidispersion index (PDI), and encapsulation efficiency of clove oil nanoparticles were resumed in Table 1. The clove oil nanoparticles at a 10% ratio of clove oil-PEG dissolved in distilled water had a Z-average size of 179 nm, PDI of 0.24, and an encapsulation efficiency of 77%. The 10% ratio of clove oil-PEG showed the best relationship between the 3 variables analyzed; *i.e.*, had a low PDI (<0.3), small size, and a high clove oil encapsulation efficiency. There are different results between nanoparticles obtained by TEM and dynamic light scattering (DLS). The size results by TEM is revealed to be smaller than the size by DLS (Fig. 1 and Table 1). The size distribution is shown in Fig. 2. Two replication of nanoparticle solutions showed bimodal (Fig. 2A and 2B) and one other replication showed unimodal size distribution.

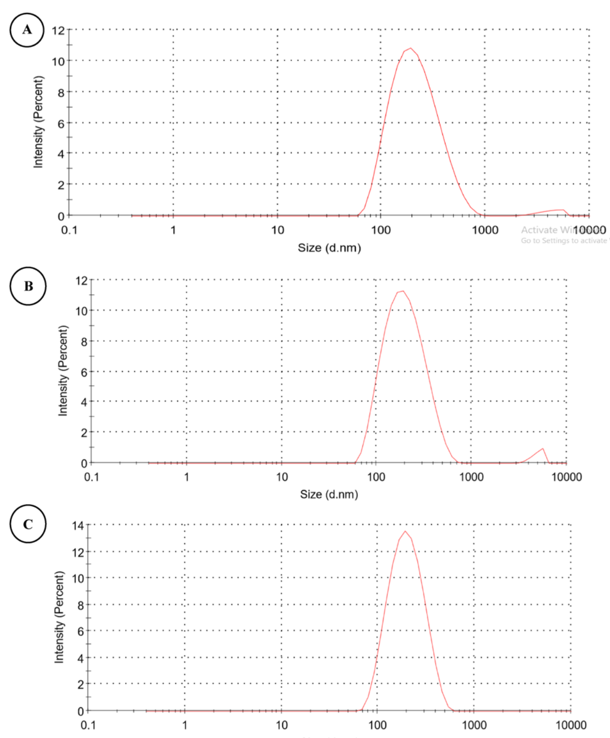


Fig. 2. The size distribution of polyethylene glycol (PEG) coating nanoparticles loaded with clove oil as measured by dynamic light scattering (A, B, and C for replication 1, 2, and 3, respectively).

Table 2. Component analysis of major constituents of clove oil pre-/postnanoformulation by gas chromatography-mass spectrometry (GC-MS) (%)

	Eugenol	*trans*-Caryophyllene
Free clove essential oil	92.43	100
Clove oil nanoparticles	7.57	—

3. Chemical composition clove oil pre/post-nanoformulation

This clove oil comprises two main components at pre-nanoformulation, eugenol, and *trans*-caryophyllene (Table 2). The main compound of clove oil before the formulation is eugenol and this compound was maintained and increased after formulation. For the *trans*-caryophyllene compound, there is a reduction in content after the encapsulated process (Table 2).

4. Residual contact toxicity of clove oil and clove oil nanoparticles

The residual effects of clove oil nanoparticles to *T. castaneum* at 14.6% concentration (equal to 1.15% clove oil) and 15.2% concentration (equal to 1.20% clove oil) are shown in Fig. 3 (data mortality obtained after 168 hr exposure). Clove oil nanoparticles produced contact toxicity for 16 weeks. At 14.6% concentration after 16 weeks of storage, nanoformulation still produced more than 70% and 90% mortality at 15.2% concentration. In this experiment, results of residual contact toxicity showed a reduction of the toxicity of clove oil nanoparticles in all of the concentration level compared with free clove oil for 16 weeks of storage, which toxicity of clove oil from the lowest to highest concentration caused 100% mortality after 24 hr of exposure.

For the evaluated fumigant activity, the results showed that clove oil was able to cause 100% mortality after 24 hr of exposure while clove oil nanoparticles, did not cause any effect after 120 hr of exposure. This could be indicating that nanoformulation reduces the volatility of constituents from clove oil. The LC_{50} values of clove oil nanoparticles for the adult of *T. castaneum* obtained from contact toxicity data after 3 and 5 days of exposure are informed in Fig. 4. Because for free clove oil treatment at all concentrations, occur 100% mortality during 16 weeks storage, so, the LC_{50} value can't be analyzed. Based on this, it can be concluded that the LC_{50} value of clove oil is smaller than the clove oil nanoparticles.

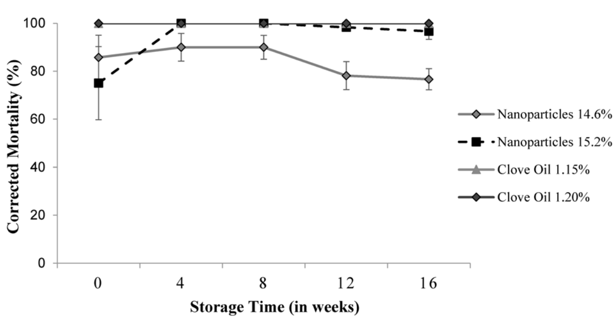


Fig. 3. Contact toxicity of clove oil and clove oil nanoparticles at 14.6% (equal to 1.15% clove oil) and 15.2% (equal to 1.20% clove oil) after 168 hr exposure against adults of *T. castaneum*. The error bar is the standard error.

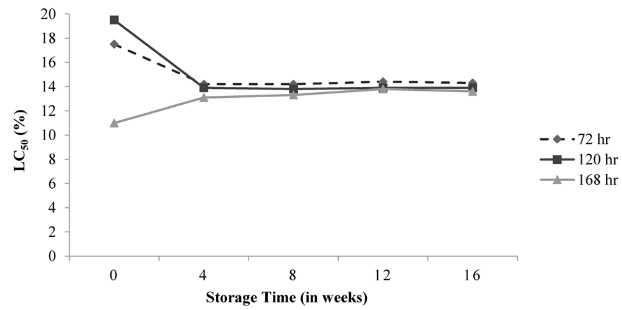


Fig. 4. LC_{50} values from clove oil nanoparticles against adults of *T. castaneum* (data mortality obtained after 72, 120, and 168 hr exposure).

Discussion

The concept of shape may seem intuitive until one attempts to quantify any but the most symmetric of particle systems.[18] The clove oil nanoparticles appearing has irregular shapes in good dispersion (Fig. 1B) different from another nanoparticle system that similarly used PEG as coating, but loaded with garlic essential oil which they appeared round.[17] This difference may be due to the different types of essential oil being evaluated. The shape is an important factor in the biological response, which can affect the toxicology of a nanoparticle.[18–20] For those particles that do not have readily definable shapes (such as uniform spheres and rods) many 'shape factors' have been devised to attempt to quantify them. Several of the most useful include sphericity or circularity, aspect ratio, elongation, convexity, fractal dimension and others,[18] so further studies are required to know the 'shape factors" in this nanoparticle.

In this study, used a 10% ratio of clove oil nanoparticles to characterize the size, the clove oil content, chemical composition and to evaluate the residual contact toxicity because this is an optimal ratio to nanoformulation.[21] Clove nanoparticles had a low PDI (<0.3), small size, and a high clove oil encapsulation efficiency (Table 1). The quantity of encapsulated chemical depends on its concentration visà-vis the polymer. In poly-ε-caprolactone nanoparticles containing griseofulvin case, the optimal percentage of a chemical compared with polymer was found to be 9%.[22]

Three factors are deemed essential in the ultimate determination of nanoparticle size, that is the concentration of polymer in the organic phase, the polarity of the solvents, and the internal or external phase ratio.[22] The difference in size between nanoparticles obtained through TEM (Fig. 1B) and dynamic light scattering (DLS) (Table 1), in which the results used TEM were smaller than used DLS. It could be caused by the preparation process for TEM. During the preparation process of visualizing an image on the nanoparticles, the heat generated by electron beams could be caused by the melting of PEG, which may have softened and melted the PEG wall.[17] The size average of nanoparticles also different from the previous result,[23] which in this study was better because smaller. This is probably because during the grinding process, in this study it was more optimal than the previous study, such as the duration of the grinding process is optimal. The grinding time and the moisture content had significant influences on the formation of nanoparticles.[24]

The PDI is a measure of the size distribution of nanoparticles. Clove oil nanoparticles have a polydispersity index of 0.24 which indicates that nanoparticles have a rather narrow size distribution and distribution type include in moderate polydisperse (0.1–0.4).[25] This type tends to be enough narrow with a good level of uniformity.[21] This system tends to be stable compared to the broad polydisperse type because this type tends to form more aggregates.

Encapsulation efficiency is one of the important factors for nanoparticle formulation, which good nanoparticles should have a high percentage of encapsulation efficiency. Oil-encapsulation efficiency in this study reached 77%. The results show that the solid dispersion method used PEG could be used to formulate clove essential oil as nanoparticles and feasible, with a high encapsulation efficiency.

The CO-NPs revealed a bimodal (Fig. 2A and 2B) and unimodal (Fig. 2C) size distribution, although in bimodal size distribution showing two peaks the intensity of the second peak was very low (under 10%) and the peak is edge peak distribution. This means is an additional, out-of-place peak at the edge of the distribution and the data set is an outlier. This result is the same as the previous result that also showed clove oil nanoparticles size distribution is bimodal.[23]

Analysis of the results from GC-MS indicates there were no significant chemical variations between pre-encapsulated and post-encapsulated, with a composition of two main components, namely eugenol and *trans*-caryophyllene (Table 2). This means, there was no oxidized found, except in the variations of the content ratio of major constituents. The volatility and diffusion of the core material through the wall of the PEG can be caused by environmental factors, for example, temperature, light, and oxygen. These factors can catalyze reactions, leading to a decrease in the content of the compound originally encapsulated.[26] On the other hand, the decreasing also a function of the susceptibility of the core material to degradation processes, including principally oxidative and dehydration reactions.[27] Most essential oils components relatively highly volatile substances and chemically labile components as a result of oxidation, chemical interactions, or volatilization, including eugenol which is easily damaged.[28] The results further indicate that nanoparticles containing clove essential oil are stable when encapsulated by PEG, mainly for the eugenol compound.

Clove oil nanoparticles produced high contact toxicity during 16 weeks of storage (Fig. 4). This could be indicating there is an extension of clove oil release that could be achieved by nanoformulation. However, for nanoparticles treated group, contact toxicity decreased gradually with the longer storage period, but the speed of insecticidal loss became slow, indicating that nanoparticles slowly and continuously release active compounds.

Nanoparticles can exhibit different properties compared with their bulk counterparts.[29] The reduction of clove oil nanoparticles toxicity in this research was similar to Hyari *et al.* (1977) that showed the encapsulated and emulsifiable formulations of malathion and fenitrothion do not increase effectiveness for several storage pests.[30] This results different from the results of Yang *et al.* (2009) and Gonzales *et al.* (2014) with the same nanoparticle system but different in essential oil was used, Yang *et al.* (2009) used garlic essential oil and Gonzales *et al.* (2014) used geranium and bergamot oil for nanoparticles, which showed the increasing of contact toxicity to *T. castaneum*.[17,21]

Loss of fumigant property could be indicating that nanoformulation reduces the volatility of constituents from clove oil. This result is similar to other nanoparticles systems, in PEG nanoparticles containing geranium or bergamot essential oil, the coating also reducing fumigant properties.[21] In solid lipid nanoparticles of *Artemisia arborescens* oil, nanoparticles were able to reduce the rapid evaporation of essential oil if compared with the reference emulsions.[31]

Reduction of contact toxicity, when compared with free clove oil, was probably due to the main absorption route involved not only one route, such as direct contact with the cuticle but from the many absorption routes involved together, such as the respiratory pathway as fumigants. In this study showing nanoparticle formulation caused the loss of fumigant property. There is also the possibility of loss or reduction of other properties (such as repellent and antifeedant). Changes in the insecticidal activity of nanoparticles also could be attributed to variations in the main composition of post-encapsulated essential oil.[17] The toxicity of the mixture of the component shows a synergistic effect among the constituents, with all the constituents needed for the full toxicity of natural oils.[32,33] There are synergistic effects of three types of oils containing eugenol, α-terpineol, and cinnamic alcohol, where the octopaminergic system mediates the activity of eugenol. In this study, there was a reduction in the *trans*-caryophyllene compound, could be indicated that the combination of eugenol and *trans*-caryophyllene had stronger toxicity than individual compound against *T. castaneum*.

The toxicity of clove oil nanoparticles was time depending, which decreasing during the experiment, indicated by increasing of LC_{50}. Besides, with the nanosize characteristic, nanoparticles may not affect the physical properties of grains when mixed, because will be reduced the friction between grains and nanoparticles. Furthermore, with its water-soluble characteristic, particles can be easily washed using water. Thus, clove oil formulation has the potential to be applied directly mixing with stored products in the protection of stored products against storage pests.

Solid dispersion using PEG is one of the most promising strategies to improve the bioavailability of materials such as plant-based insecticides which are difficult to dissolve in water. Polyethylene glycols are a polymer that is commonly used because of its high hydrophilicity and can improve the biocompatibility of nanoparticles.[34] This polymer has characteristics: good solubility in many organic solvents, have a low melting point (below 65°C), low toxicity, able to dissolve some compounds, and could improve compound moisture.[35,36] The addition of a material (such as essential oil) or the fast cooling of the melted PEG could act as an inhibitor of crystallization resulting in a higher percentage of amorphous and imperfectly crystalline material; the amorphous character is common with polymeric molecules used as a carrier.[21]

However, further evaluation is needed on the other biological activities, for example, evaluation of antifeedant and enzyme activity that plays a role in the toxicity process. Economical evaluation of the cost in large storage also is needed.

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