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Activity of n-hexane Compounds and Ethyl Acetate of Gaharu Leaf (*Aquilaria malaccensis)* to Control of *Coptotermes curvignathus* Ground Termites with Nanoparticle Technology

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Abstract

Secondary metabolites of gaharu leaves (*Aquilaria malaccensis*) is potentially as natural anti-termites. However, the ability of gaharu leaf metabolite to control termite attack on the building still has not shown optimal results. This is due to the large secondary metabolite size so that nanoparticle technology is used in this study to minimize the size of secondary metabolites. This research was divided into two stages. The first stage of the preliminary study consisted of sample preparation, extraction, and partition of gaharu leaf (*Aquilaria malaccensis*), making n-hexane nanochitosan and ethyl acetate nanochitosan, and sample characteristic test including phytochemicals testing, particle size testing with *Particle Size Analyzer* and *Scanning Electron Microscopy*. The second stage is anti-termite activity test with the method of forced feeding test. The test was performed for 7 days

with variation of 0% concentration (negative control), 2%, 4%, 6%, 8%, 10% and 0.25% of fipronil (positive control). The results showed that the crude extract of methanol, nanochitosan ethyl acetate, and n-hexane nanochitosan could reach 100% termite mortality within 7 days of testing. The extract that had the highest mortality was found in n-hexane nanocitosan because it achieved 100% mortality at 4% concentration. The termite mortality data was also proportional to the LC_{50} value held by each extract. N-hexane nanochitosan has the lowest LC_{50} value of 0.11% followed by 0.88% ethyl acetate nanochitosan, and 5.88% of methanol extract. Particle size nanochitosan n-hexane gaharu leaf is 16,3 nm and nanochitosan ethyl acetate gaharu leaf has a particle size is 26,6 nm.
Keywords: Coptotermes, Nanoparticle, Pest Control, Technology, Termite

1.Introduction

Termites are reported to have attacked many buildings in Indonesia, especially from the subterranean termites (ground termites) namely *Coptotermes curvignathus* Holmgren. [1] The high frequency of termite attacks in major cities is thought to be due to several factors such as the rate of urban development, the abundance of ground termites of the *Coptotermes* genus, which acts as the main building pest and environmental conditions that support termite life.[2]

The economic losses caused by termite attacks on buildings continue to increase from year to year.[3] Total economic loss due to termite attack reached USD200-300 billion.[4] The average percentage of termite attack on residential buildings in big cities such as Jakarta, Surabaya, Bandung and Batam reach more than 70%, the value of termite losses each year increases by about.

The problem of controlling termites has not been solved until now due to the fact that the chemical preservatives in the market are aimed at individual species of termites and therefore are ineffective. No method destroys these insects quickly or definitely.[5] Termiticides such as organophosphates, pyrethroids, phenyl pyrazols and nitro guanidine are very effective at low doses, but are not selective to target organisms, contaminate water and water sources and are difficult to decompose in the soil.[1] Due to unwise use of pesticides, many adverse effects exist including: pest resistance and resurgence, secondary pests, death of natural enemies, environmental damage and human harm. It is therefore necessary to control alternative termites that are natural and do not cause environmental pollution, like anti-termite of n-hexane and ethyl acetate compounds of gaharu leaves (*Aquilaria malaccensis*) with nanoparticle technology.

Gaharu leaves have phytochemical that can be used as termite controllers.[6] The ability of gaharu leaf extract in destroying termites is supported by the content of secondary metabolite compounds such as alkaloid, flavonoids, triterpenoids, steroids, tannins, and saponins group compounds in the extract.[7,8] However, the ability of gaharu leaf metabolite to control termite attack on the building still has not shown optimal results. This is due to the large secondary metabolite size so that nanoparticle technology is used in this study to minimize the size of secondary metabolites so as to expand the surface area and facilitate the absorption of materials to the building.

This nanoparticle technology used chitosan. Chitosan that was not soluble in alcohol was made into nanochitosan where its solubility will be greater in alcohol. Micro/ nanoparticles of chitosan have many advantages such as non-toxic, stable during use, wide surface area, and can be used as matrices for various types of drugs and plant extracts.[9] Chitosan has several beneficial properties such as anti-microbial, wound healing, non-toxic, cheap, biocompatible, biodegradable and water soluble. Therefore, it is expected that the results of this study can be used as an environment-friendly termite controlling solution and have better capability than synthetic chemicals.

2.Materials and Methods

Sample Preparation

Gaharu leaves (*Aquilaria malaccensis*) were obtained from Samarinda Forest. Gaharu leaves of 5 kg were cleaned of dirt follow-up and separated from the trunk and then air-dried at room temperature. The leaves were then mashed using a grinding machine and 40 mesh sieve. The result was stored at room temperature in a sealed container.

Sample Extraction and Partition

The 260 g of gaharu leaf powder was extracted and partitioned using *sokletasi* method with methanol, n-hexane, and ethyl acetate solvent to obtain methanol fraction, n-hexane fraction, and ethyl fraction of gaharu leaves 250 ml each. The obtained results were stored at room temperature in a sealed container.

Phytochemical Screening

Testing of the active components contained in this extract was carried out qualitatively by phytochemical test methods including alkaloid, tannin, flavonoid, saponin, steroid and triterpenoid tests.

a. Alkaloids

A total of 1 mL of extract was added 4 drops of 2 N sulfuric acid were then tested with three alkaloid reagents such as, Dragendorff, Meyer, and Wagner reagents. The test results were tested positive when Meyer's reagents formed yellowish white deposits, brown precipitates on Wagner reagents, and red sediment on Dragendorff reagents.[10]

b. Polyphenols / Tannins

The extract solution was added 2 ml of 2% FeCl3 solution. Blue-green or black color indicates the presence of polyphenols / tannins.[11]

c. Identify Flavonoids

A total of 1 mL of extract was added magnesium powder of 0.10 mg and 0.40 mL of amyl alcohol (37% hydrochloric acid mixture and 95% ethanol with the same volume), and 4 mL of alcohol and then the mixture was shaken. The red, yellow or orange color formed on the amyl alcohol layer indicates the presence of flavonoids.[10]

d. Identification of Saponin

The extract solution was added with aquades, then was shook vigorously. Saponin-containing solutions will produce stable 1 to 10 cm foams and not less than 10 minutes.[10]

e. Steroid / Triterpenoid

Samples were added 10 drops of glacial acetic acid and 2 drops of concentrated sulfuric acid. The solution was shook gently, left for several minutes. Steroids provided blue or green, while the triterpenoid provided red or purple.[10]

2.4 The making of Chitosan Nanoparticles[12]

Chitosan was made of 3% concentration using ionic gelation method. A total of 3 grams of chitosan were dissolved in 100 mL of 1% acetic acid with magnetic stirring at room temperature. The soft gel bonding was performed using a magnetic stirrer for 2 hours at 2000 rpm. Then, Tween 80 0.1% solution of 20 uL was added, which aimed to separate gel one with another, then it was made homogenous in magnetic stirrer for 30 minutes, aiming to keep the resulting particle size stable. The encapsulation process began with a solution of nanochitosan was added with 100 mL gaharu leaf extract and homogenized using a magnetic stirrer for 45 minutes.

Characterization of Nanochitosan of Gaharu Leaf Extract

The tests performed for the nanochitosan extract in this study were determining the sample morphology by *Scanning Electron Microscopy* (SEM) test, sample particle size analysis by *Particle Size Analyzer* (PSA) test.

a. PSA (*Particle Size Analyzer*)[13]

The particle size test was performed using PSA (*Particle Size Analyzer*) testing. The sample of the solution was taken with a pipette and then inserted into a tube with a maximum height of 15 mm. Test results would appear on the computer screen.

b. SEM (*Scanning Electron Microscopy*) Test[14]

The encapsulated extract of nanochitosan was placed on a 1 cm diameter of brass piece using two-sided masking tape. Then the powder was made to be electrically conductive by using light from a thin layer of coating for 30 seconds under 2 Pa and 10 kV electron voltage with 500x, 2500x, and 5000x magnification.

Termite Activity Test

Anti-termite activity of gaharu leaf extract was tested using force feeding test with some modifications.[15] Test glass made of plastic material (diameter of 5 cm, height of 5 cm). The bottom of the test glass was a porous plaster of paris and sterile sand of 10 g.

The feed paper (diameter of 2 cm; Whatman paper No. 41) was stirred at 60° C for 12 hours, kept in desiccator for 24 hours, then weighed for initial weight. After that, the feed paper was immersed in a nanochitosan solution for 1 hour with 0% (negative control), 2%, 4%, 6%, 8%, and 10% (v/v) concentration. Positive control used was Regent brand fipronil with concentration of 0,25% (2,5 mL in 1 L of water), whereas there was no treatment at negative control of addition of extract (solvent only).

After soaking, the feed paper was allowed to evaporate the solvent. The 4 cm (modified) diameter of strimine cloth was placed in a test glass and on a strimine cloth was placed the feed paper. Test glass was placed in a container with wet cotton in its bottom. A total of 30 active termites consisting of 25 worker castes and 5 warrior castes were inserted into the test glass. The test glass was covered with a black cloth and kept in a place protected from light for 7 days. The feed paper was stirred at 60°C for 12 hours and stored in the desiccator for 24 hours, then the paper was weighed for final weights. Testing was done with four repetitions. The mortality observation was performed by counting the number of termites that died every day for 7 days. The accumulated amount was calculated on the 7th day. The decreased weight percentage (PB) of feed paper was calculated after 7 days.

Data Analysis

The percentage data of mortality were analyzed by analysis of variance (ANAVA) at 95% confidence level and got different result so that it was continued with Tukey test. The estimated value of 50% mortality (LC_{50}) was determined using the regression line equation between the concentration log and the probit of death (probit analysis).

3.Results and Discussion

Phytochemical Screening

Table 1. The screening result of Phytochemical Extract and Gaharu Leaf Faction

Uji	Metanol extract	Fraction	
		Fraction n-hexane	Fraction ethyl acetate
Alkaloid	+	+	+
Polifenol/Tanin	+	+	+
Flavonoid	+	+	+
Saponin	-	+	+
Steroid	-	+	+
Triterpenoid	+	-	-

The results of phytochemical screening (Table 1) showed that the gaharu *Aquilaria malaccensis* leaf sample contained of alkaloids, polyphenols / tannins, flavonoids, saponin, steroids and triterpenoids. The rough extract of gaharu leaf methanol showed positive results on alkaloids, polyphenols / tannins, flavonoids and triterpenoids. The n-hexane fraction and ethyl acetate fraction showed positive results in the alkaloids, polyphenols / tannins, flavonoids, saponin, steroids.

The rough extract of methanol, methanol faction, and ethyl acetate faction showed positive results on the polyphenol / tannin test in the presence of the color change of the extract solution to blue-green or black. A positive ethyl acetate and n-hexane faction contained steroids that give a green color to the extract solution. The methanol extract provided a reddish-brown discoloration that indicated the presence of a triterpenoid compound. The faction of n-hexane and ethyl acetate showed a high and stable foam for 10 minutes.

Based on Table 1, it can be seen that the methanol extract, the n-hexane faction, and the ethyl acetate faction of gaharu leaves contain alkaloids and tannins. The highest compound content of gaharu leaves was alkaloids, of 0.148%.[6]

Nanochitosan Anti-Termite Activity of Gaharu Leaf *Aquilaria malaccensis*

The bioavailability of gaharu leaves can be seen from termite mortality calculated after 7 days of treatment. Based on the data in Table 2, it shows that the greater the concentration of extract given on termites, the greater the value of termite mortality percentage. This was because large concentrations have more extractive substances that are more toxic than smaller concentrations.

Table 2. Termite Mortality After 7 Days of Feeding

Extract	Average Mortality of Termites (%)*					
	Concentration (%)					
	0	2	4	6	8	10
Metanol	10 ± 3	30 ± 3	48 ± 1,73	54,33 ± 2,3	60 ± 3	80 ± 3
N. Etil Asetat	9 ± 7,2	76,67 ± 3,51	86,67 ± 3,51	92 ± 1,73	96 ± 0	100 ± 0
N. n-Heksana	12,33 ± 5,03	89 ± 1,73	100 ± 0	100 ± 0	100 ± 0	100 ± 0

*Values are means ± standard deviations from three replications. N= Nanokitosan

The rough extract of methanol, ethyl acetate nanochitosan, and n-hexane nanochitosan can reach 100% termite mortality within 7 days of testing (Table 2). This proved that gaharu leaf extract has bioactivity as a very strong anti-termite.

The extract that had the highest mortality was found in n-hexane nanochitosan because it achieved 100% mortality at 4% concentration. Termite mortality occurred because there was no termite interest in the food provided and the absence of other food alternatives.[16] Positive control (0.25% fipronil, *Regent brand*) caused 100% mortality on the first day. This concentration was much smaller than that of ethyl acetate nanochitosan and n-hexane nanochitosan. Thus, the use of fipronil termiticide is still much more effective and efficient.

Termiticides fipronil is more effective and efficient, but nanoparticle chitosan has a lot of advantages such is a not toxic, stable during use, wide surface area and can be used as a matrix for different types of drugs and plant extracts.[9] Chitosan has a beneficial properties of anti-microbial in nature such is wound healing, non-toxic, inexpensive, biocompatible bonding agent, can dibiodegradasi, and water-soluble.

Table 3. Results of Two-Way ANOVA Calculations Using SPSS 23.0 Program

F	df1	df2	Sig.
3,116	17	36	0,002

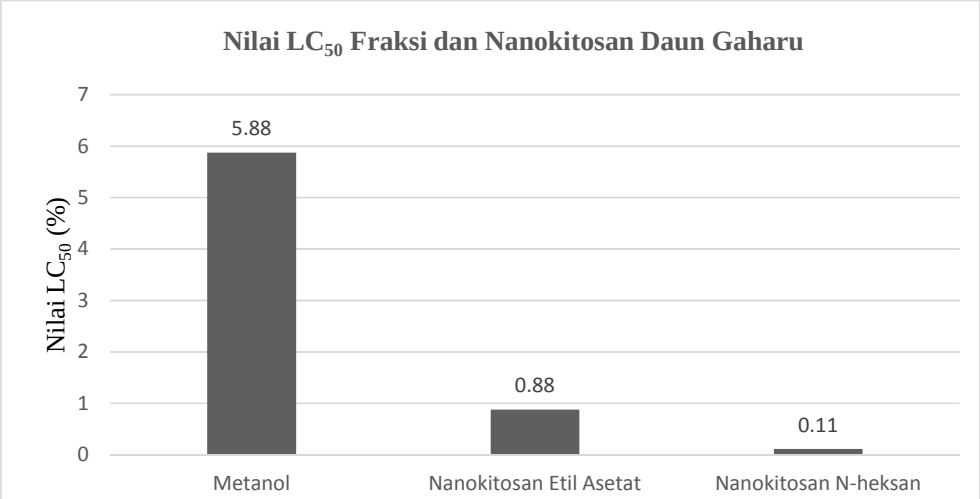
The result of ANOVA calculation in Table 3 shows that the concentration factor significantly shows significantly different result at 95% confidence level. Negative controls caused mortality <15%. Tukey test results showed that the mortality of termite control treatment (0% concentration) was significantly different with the extract treatment of 2%, 4%, 6%, 8% and 10%. This indicates that the addition of gaharu leaf extract has an effect on termite mortality. Mortality that occurred in the negative control was suspected because termites still cannot adapt to the new environment that was test glass.

Figure 1. LC_{50} Extract and Nanochitosan of Gaharu Leaves

The termite mortality data was also proportional to the LC50 value held by each extract (Figure 1). The LC_{50} value indicated a concentration level that can kill 50% of the number of test animals. The lower the LC_{50} value possessed by an extract, the higher the level of toxicity of the extract.

The data from Figure 1 shows that n-hexane nanochitosan had the lowest LC_{50} values of 0.11% followed by 0.88% of ethyl acetate nanochitosan and 5.88% of methanol extract. However, the value of LC_{50} owned by both nanochitosan of n-hexane was still very far from the value of LC50 fipronil, which was 24.3 × 10-4%.[17] This was due to the fact that fipronil has a central nervous system disturbing mechanism that causes interference with chloride ions through *Gamma Amino Butyric Acid* (GABA) in insects.[1]

The bioactivity of n-hexane nanochitosan was higher than that of ethyl acetate nanochitosan because it contained classes of other compounds other than steroids that reinforce its activity as anti-termite, such as flavonoids and polyphenols / tannins (Table 1). Termites avoid paper feeds containing antioxidant extractive substances, as they may interfere in the lignocellulosic digestion process by termite symbionts.[18] Various pure compounds of flavonoids in which all compounds exhibit anti-feedant activity (feeding inhibition).[15] Polar flavonoid compounds can penetrate the polar



peptidoglycan in bacterial cells that can cause cell damage. Peptidoglycan can also be damaged by the presence of tannin / polyphenol compounds that play a role in breaking the peptidoglycan bond.[19] Phenolic compounds are capable of forming complexes with proteins (cell membrane constituents) through hydrogen bonding,[10] so that toxins from extractive substances can enter the cells and attack the nucleus causing the termite nervous system to be disturbed.

Bioactivity of extractive substances to termite mortality is also supported by the termite trophalaxis properties, such as activity in the form of licking, kissing, and rubbing the limbs of individual termites to deliver food from worker caste to other colony members and sharing protozoa for individuals who have just changed skin.[1] Foods containing toxins from gaharu leaf extractive substances will be spread through trophalaxis so that it can cause mortality in a termite colony.

Particle Size of Nanochitosan Gaharu Leaves *Aquilaria malaccensis*

The assessment of particle in modern way usually uses figure anlysis of types of particle assessment such as *Particle Size Analyzer* (PSA). PSA analysis aims to find out particle size of gaharu leaf extract with the nanochitosan particle size of gaharu leaf extract.

Based on the test result of PSA, it shows that the average size of methanol geharu leaf extract is 3124,9 nm whereas the average size of nanochitosan n-hexane is 16,3 nm and the size of nanochitosan ethyl acetate of gaharu leaf extract is 26,6 nm. Nanoparticle was the particle which was solid form and the size was about 10-1000 nm. The smaller particle from 300nm can penetrate into the cells easily. [20]

Preparation methods were very influenced in the technology of making nanoparticle. Minimizing the size with magnetic stirrer can produce more stable particle with the more equally size, under 1000 nm.[21] The influence of minimizing particle size with magnetic stirrer on the high speed could generalize accepted energy in all solution, so that the size of nanoparticle was more homogenous.[22] Increasing tripoliposphat in the right way can decrease nanoparticle size and increase the power of chitosan matrix so it made nanoparticle stronger and hard to break.[23] Chitosan solution which have mixed with tripolipospat and it was added with Tween 80. Adding Tween 80 as surfactant aimed to stabilize particle emulsion in the solution by avoiding agglomeration between the solution.[24] The chitosan particles in the solution was covered and stabilized one another because there was surfactant, so that the slitting particle will more effective and the agglomeration was not occurred.

Morphology of Nanochitosan Gaharu Leaf *Aquilaria malaccensis*

Nanochitosan can be differentiated as visual by using *Scanning Electron Microscopy* (SEM). The principle of SEM was the typical wave of the electron as the diffraction from the very small corner.[25] The SEM test result of 500x magnification shows that nanochitosan n-hexane of gaharu leaf and nanochitosan ethyl acetate have the particle like roundness ball. Nanochitosan shows the less homogeneous of the particle. Nanochitosan which contained ketoprofen had roundness shape. The different size of particles is considered that it is because ketoprofen does not only penetrate into the matrix nanochitosan, but it sticks in the surface.[26]

Chitosan nanoparticle which was obtained by using magnetic stirrer result spreading the energy that tent to be equal so that in certain time, distribution of small particles can be more homogeneous and have roundness morphology.[28] That empty nanoparticle chitosan had wrinkles and flat form, whereas the Nano chitosan which was filled by ketoprofen had roundness form.[26]

Conclusion

According to the result which has been conducted, it can be concluded that nanochitosan n-hexane and ethyl acetate of gaharu leaf have potential to control the termites *Coptotermes curvignathus*. Nanochitosan n-hexane has the highest mortality of extract because it can reach 100% mortality on 4% concentration.

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