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The effectiveness of clove oil, its fractions, and clove oil-based fumigant tablet formulations, against *Tribolium castaneum* (Herbst)

Keefektifan minyak cengkeh dan fraksinya, serta formulasi tablet fumigan berbasis minyak cengkeh terhadap *Tribolium castaneum* (Herbst)

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ABSTRACT

Indonesia has seen an increase and widespread reports of resistance among stored-product insect pests to phosphine. The use of *Syzigium aromaticum* (clove oil) as an alternative fumigant may be a useful strategy to control infestation by phosphine-resistant varieties of stored-product insects. The objective of this study was to examine the effectiveness of whole (unfractionated) clove oil as well as its component fractions as a fumigant and repellent against the red flour beetle (*Tribolium castaneum* (Herbst)), and to develop a simple fumigant formulation for this purpose. The experimental design used to test the effectiveness of clove oil and its fractionation was a completely randomized design (CRD). Meanwhile, testing the effectiveness of tablet formulations was carried out by factorial CRD. Fumigation test results gave LD_{50} and LD_{95} values of 0.234 and 1.142 ml/l respectively, for crude clove oil used in a fumigation chamber against *T. castaneum*. An *n*-hexane fraction of clove oil tested under the same conditions was more lethal, causing 95% mortality of *T. castaneum* at the dose of 0.801 ml/l during fumigation. Finally, tablets containing a set proportion of clove oil and naphthalene (1:1) reached LD_{91} against *T. castaneum* after 7 days' fumigation.

Key words: fractionation, mortality, naphthalene, stored product insect

ABSTRAK

Kasus resistensi serangga hama gudang terhadap fosfin telah berkembang sangat luas di Indonesia. Penggunaan minyak cengkeh sebagai fumigan alternatif untuk mengendalikan strain resisten serangga hama gudang terhadap fosfin merupakan salah satu potensi yang baik untuk dikembangkan. Penelitian ini bertujuan untuk menguji keefektifan minyak cengkeh dan hasil fraksinasinya terhadap *Tribolium castaneum* (Herbst) dan untuk mengembangkan formulasi fumigan sederhana berbahan minyak cengkeh. Rancangan perocabaan yang digunakan untuk pengujian keefektifan minyak cengkeh dan fraksinasinya adalah rancangan acak lengkap (RAL). Sementara itu, pengujian keefektifan formulasi tablet dilakukan dengan RAL faktorial. Nilai LD_{50} dan LD_{95} minyak cengkeh kasar terhadap *T. castaneum* adalah 0,234 dan 1,142 ml/l ruang fumigasi. Fraksi *n*-heksana cengkeh lebih efektif dibandingkan dengan minyak kasarnya yang menyebabkan

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95% mortalitas *T. castaneum* pada dosis 0,801 ml/l ruang fumigasi. Tablet minyak cengkeh yang mengandung campuran minyak cengkeh dan naftalen (1:1) menyebabkan 91% mortalitas terhadap *T. castaneum* dengan lama waktu fumigasi 7 hari.

Kata kunci: fraksinasi, mortalitas, naftalen, serangga hama gudang

INTRODUCTION

Quality of post-harvest handling and storage for durable agricultural commodities constitute a major ingredient of the food security of a nation. Insect pests pose the greatest threat to post-harvest stored grains and so undermine the ability to maintain necessary levels of food security. Post-harvest losses caused by insect pests can reach 10–60% in countries where modern storage technologies are not yet fully adopted (Shaaya et al. 1997). The magnitude of stored product losses varies, depending on insect pest species, pest control method applied, duration of product storage, and the security of storage facilities against infestation.

Stored-product pests are most commonly controlled via fumigation. Since the ratification of the Montreal Protocol of 1992 on Substances that Deplete the Ozone Layer, the use of methyl bromide was banned, and phosphine has become the only option as a fumigant against insect pests of stored products. As a consequence, insect resistance to phosphine is now a global issue, causing pest control failures reported in many countries (Taylor 1989; Collins et al. 2002). New fumigants such as carbonyl sulphide (Desmarchelier 1994) and ethane dinitrile (Ryan et al. 2006) have been investigated as alternatives for food and non-food commodities. However, the potential for residues produced from fumigants containing sulfur is higher, so it is not safe to be applied to food commodities. Meanwhile, phosphine has been known as a fumigant whose residue is almost non-existent in the applied commodity. The maximum residue of phosphine found in wheat only reached 0.046 ppm and the amount decreased to 0.006 ppm after aeration (Bruce et al. 1962). Therefore, phosphine is the only fumigant product that is safe for food and food products.

Essential oils represent a potential and less toxic alternative to phosphine as a fumigant. Plant-based oils have advantages over conventional fumigants including low mammalian toxicity, rapid degradation, and local availability. Many recent studies have proven that these substances composed of volatile complex compounds could be effective against various species of stored-product insect pests (Mahmoudvand et al. 2011; Manzoomi et al. 2010; Rani 2012; Tunc et al. 2000). Indonesia is a tropical country with highly diverse and abundant plant species, some of which may contain essential oils that could serve as alternatives to conventional pesticides. It is important to research local plant sources and their potential effectiveness when used to produce fumigants for controlling stored-product insects.

Essential oils generally contain hundreds of different constituent chemicals, but certain components will be dominant and present in higher proportions (Rajendran & Sriranjini 2008). Analysis and chemical separation of essential oil compounds is needed to identify any active components, and these are more effective as pesticides when isolated and concentrated for that use (Kim & Ahn 2001). Identification of effective plant-based fumigants begins with a series of studies assessing the effectiveness of different essential oils as pesticides. This entails fractionation, analysis of chemical compounds, and development of essential oil formulations that can be used to control phosphine-resistant strains of stored product insects. In this research, an exploration of clove oil (*Syzigium aromaticum*), both crude and fractionated, was carried out to determine its toxicity to insect stored product pests. Previous studies have shown that fractionation of peppermint, cinnamon, and cardamom oils can increase their toxicity to *Tribolium castaneum* (Herbst) insects (Asnan et al. 2018).

A series of studies on exploration and development of formulation of essential oils as fumigants to control stored-product insects are needed to carry out to obtain alternative fumigant than can be used to control phosphine resistant strains of stored product insects. In addition, essential oil compounds that are proven effective against stored-product pests can be a model for new

synthetic fumigants that are more environmentally friendly with low mammalian toxicity. Although essential oil has the most promising properties as a biofumigant, problems related to their volatility must be resolved before being used as an alternative fumigant for pest management. Therefore, this research also developed a simple fumigant formulation with food-grade ingredients to ensure safety in the application and preparation of materials. It is hoped that this formulation can reduce oil evaporation and slow down the rate of degradation in the environment. The research related to essential oil-based biofumigant formulations has not been widely carried out in Indonesia. The objective of this study was to examine the effectiveness of clove oil (*S. aromaticum*) and its fractions against *T. castaneum* and to develop a simple fumigant formulation composed of any effective fractions.

MATERIAL AND METHODS

Preparation of insects test

Insect mass rearing was conducted at the Entomology Laboratory, Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP), Bogor by placing 500 adult *T. castaneum* individuals into a glass jar containing rice bran as feed. After two weeks, all adults were removed, leaving behind the bran feed and *T. castaneum* eggs. The jar was incubated for 4 weeks. The F1 generation adults emerged at age 7–14 days for use as test insects.

Fumigant toxicity of clove oil

The clove (*S. aromaticum*) oil used in this research was purchased as pure oil from Lansida Group, Yogyakarta, Indonesia. The toxicity of its oil was tested in 2 steps: a preliminary and then an advanced study (bioassay). The concentrations of clove oil used for the preliminary study were 5%, 10%, and 15% (v/v), diluted in acetone. 0.5 ml of each sample/concentration was placed onto a piece of 9 mm thick of Whatman filter paper glued to the inside of a petri dish lid, then air-dried for 2 minutes. As a control, identical filter papers were dripped with acetone only. 20 test insects were put into petri dish and covered with gauze before closing them inside using the treated petri dish lids. This prevented direct contact between the test insects and the filter paper on the inner lid (Figure 1). The petri dishes were then sealed with plasticine to prevent gas leakage. Insect mortality rates were observed at 72 hours after treatment. These treatments were replicated 5 times. The results of this preliminary study were used to identify five concentration levels with the highest mortality rates, appropriate for an advanced bioassay. Concentrations demonstrated by the preliminary results to cause test insect mortality ranged between 50% and 95%. The bioassay for advanced testing replicated the methods of the preliminary test, with treatments repeated 5 times for each of the concentration levels selected for further study. The experimental design used in this study is completely randomized experimental design.

Fractionation of clove oil and bioassay of its fractions against *T. castaneum*

Clove oil was subjected to multilevel fractionation using 3 solvents of different polarity: water-methanol (polar), ethyl acetate (semi-polar), and *n*-hexane (non-polar). 50 ml of clove essential oil was poured into a separatory funnel, with 15 ml *n*-hexane then added, followed by gradual partitioning using 15 ml water-methanol (7:3). This mixture was then shaken and allowed to settle until two separate layers of liquid formed. Each of the two layers was collected separately to obtain *n*-hexane and methanol fractions.

The methanol fraction was further partitioned via the gradual addition of 15 ml ethyl acetate, shaken again to create a mixture that would also yield 2 layers, consisting of methanol and ethyl acetate fractions. The three fractions ultimately obtained (*n*-hexane, methanol, ethyl acetate) were then concentrated using a rotary vacuum evaporator. The resulting concentrated fractions were tested for toxicity against *T. castaneum* using the same method with the toxicity test for the crude clove oil.

GC-MS analysis

GC-MS analysis was conducted to identify the chemical composition of the *n*-hexane fraction of clove oil. The GC conditions were as follows:

sample volume injected: 0.2 µl; helium carrier gas flow rate 3 ml/min at 10.523 psi pressure; injection and detector temperature 250 °C held for 8 minutes. The Library Wiley 9 standard mass-spectrum data bank was used for reference to identify any compound.

Tablet preparation and bioassay

We prepared tablets using a formulation containing the *n*-hexane fraction of clove oil created earlier, naphthalene, and Tween 80. 25 ml of the *n*-hexane fraction was emulsified using 50 ml Tween 80, with the mixture stirred or shaken until homogeny. The process was repeated with naphthalene instead of the *n*-hexane fraction, creating emulsified naphthalene. Tween 80 was used in both cases in order to facilitate mixture homogeny and fixation into 10 g talcum tablets. Four kinds of talcum tablets were prepared using a manual tablet printer. They contained the following proportions of active ingredients;

a. 3 ml of clove oil *n*-hexane fraction emulsion;
b. 3 ml of naphthalene emulsion;
c. 1.5 ml clove oil *n*-hexane fraction emulsion + 1.5 ml naphthalene emulsion;
d. 2 ml clove oil *n*-hexane fraction emulsion + 1.0 ml naphthalene emulsion.

The 4 types of essential oil fumigant tablets were then placed inside tea filter bags for testing against *T. castaneum* (Figure 2A). The bioassay of the tablets included 2 variables: (1) tablet composition (4 types described above) and (2) exposure time (3, 5, and 7 days). Each tablet type was subjected to all three exposure periods, and each treatment was repeated 5 times.

For each treatment, the protocol began with a 2-liter glass jar containing 40 g or plain uncooked rice. Twenty (20) *T. castaneum* adult insects were then introduced into the jar and a wire mesh platform placed so that it stood 10 cm above the insects and rice. Fumigant tablets were placed on the wire platform and the jars were sealed closed using plasticine to make the lid gastight. This procedure was carried out for treatments with exposure times of 3, 5, and 7, so that there were 3 treatment series based on the length of exposure time and 5 replications for each treatment. The experimental design used in this study is factorial completely randomized design with the first factor was the composition of the fumigant tablet and the second factor was fumigant exposure time. Mortality of test insects was observed at the ends of exposure time which are 3, 5, and 7 days.

Data analysis

This study used insect mortality as its sole parameter of pesticide effectiveness. Mortality data were processed using probit analysis with POLO-PC software (LeOra Software 1987), and Microsoft Excel 2007, and all data were analyzed using SAS 9.2. A comparison of mean mortality rates between the treatments was conducted with Duncan multiple range tests ($\alpha = 0.5$).

RESULTS

Fumigant toxicity of clove oil

The effectiveness of clove oil at concentrations ranging from 5–25% was tested. Clove oil at 5%

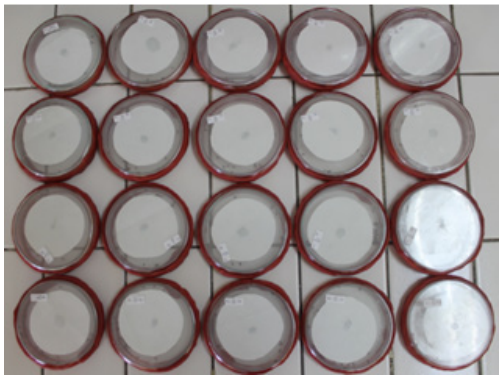

Figure 1. Petri dishes used as fumigation chambers (red rings are plasticine used to seal petri dishes cup to prevent gas leakeage).

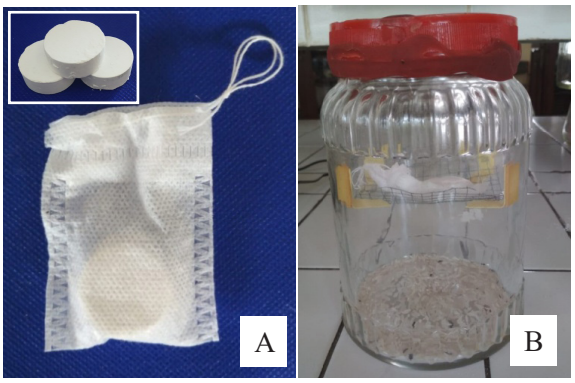

Figure 2. Clove oil-based fumigant tablets (A). Treatment of fumigant tablets in a 2-liter fumigation chamber (B).

(equivalent to 0.25 ml/l of air) and 25% (equivalent to 1.25 ml/l of air) caused 54% and 98% mortality of insect, respectively, after 72 hours treatment (Table 1). The LC_{50} and LC_{95} values of the clove oil at 5% and 25% concentration were 4.68% (±0.25 ml/l air) and 22.83% (±1.1 ml/l air), respectively.

Fractionation of clove oil and chemical composition of clove oil *n*-hexane fraction

Our process of fractionation of clove oil resulted in 4 ml ethyl acetate fraction and 95 ml of *n*-hexane fraction. Any methanol fraction was discarded. The *n*-hexane and ethyl acetate fractions were yellowish-orange. Analysis of the chemical composition of the *n*-hexane fraction of clove oil using GC-MS is shown in Figure 3. The chromatogram indicates that the *n*-hexane fraction contained two dominant compounds at the retention times 8.363 and 9.645 minutes: eugenol (76.54%) and beta-caryophyllene (19.56%). Three other components found at retention times 5.242, 8.662, and 10.329 minutes were less dominant with concentrations of less than 4%. Those less dominant compounds were methyl salicylate (0.98%), α-copaen (0.67%), and α.-humulene (2.26%).

Effectiveness of clove oil *n*-hexane fraction against *T. castaneum*

The *n*-hexane fraction was then tested for its effectiveness as a fumigant against *T. castaneum* at concentrations of 3%, 6%, 9%, 12%, and 15%. The LD_{50} and LD_{95} values of the *n*-hexane fraction of clove oil against *T. castaneum* was 0.185 ml/l fumigation chamber and 0.801 ml/l fumigation chamber, respectively. The regression equation was y = - 1.457 + 2.567x and the R^2 value was 0.9028. This value indicates that 90.28% of the

Table 1. Mortality for *Tribolium castaneum* adults treated with clove essential oil

Concentration (%, v/v)	Dose (ml/l air)	*T. castaneum* mortality ± SD[a] (%)
		72 HAT[b]
Control	-	0.00 ± 0.00 d
5	0.25	54.00 ± 10.84 c
10	0.5	80.00 ± 5.00 b
15	0.75	82.00 ± 5.70 b
20	1	95.00 ± 5.00 a
25	1.25	98.00 ± 4.47 a

[a]SD: standard deviation; [b]HAT: hours after treatment.

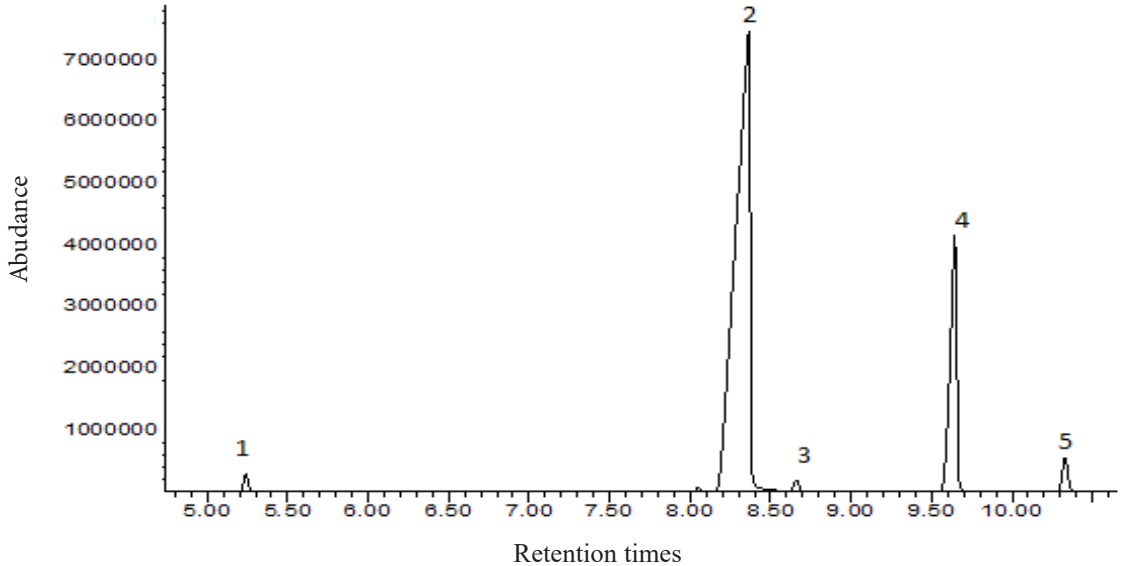

Figure 3. Chromatogram *n*-hexane fraction of clove oil. 1: methyl salicylate (0.98%); 2: eugenol (76.54%); 3: α-copaen (0.67%); 4: beta-caryophyllene (19.56%); 5: α.-humulene (2.26%).

mortality of *T. castaneum* can be attributed to the treatment at the doses tested.

Clove oil-based fumigant tablet and bioassay

The effectiveness of clove oil-based fumigant tablets against *T. castaneum* was tested using several different treatments (different tablet compositions and different exposure time). The results of the bioassays are presented in Table 2. *T. castaneum* mortality is lowest (never exceeding 50% for any exposure time) for treatment I using tablets containing clove oil only. By contrast, *T. castaneum* mortality for treatments II, III, and IV reached more than 70% at 7 days exposure time. It appears the addition of naphthalene to the tablet formulation increases the effectiveness of clove oil against *T. castaneum*. The 1:1 mixture of clove oil and naphthalene (treatment III) resulted in 91% mortality of *T. castaneum* after 7 days of fumigant exposure. This result is not significantly different from that obtained using fumigant tablets containing naphthalene only, at the same exposure duration. In this case, the length of exposure affects the death of the test insects. The longer the exposure time applied caused the higher the mortality occurred. It is because the insects inhale and are exposed to more toxic gases during the exposure time.

DISCUSSIONS

Clove oil is widely used and well known for its medicinal properties as an antiseptic and also analgesic (Abo-El-Saad 2011). In addition, the biological activity of clove oil has been investigated for agricultural use to control various insect pests. Clove oil has been shown to be effective when used to suppress progeny development of *T. castaneum* and *Sitophilus zeamais* Motsch with isoeugenol (Ho et al. 1994). Our present research results demonstrate that clove oil is an effective pesticide against *T. castaneum* and shows promise for development as an alternative fumigant to commercial products. We also find that fractionation can increase the toxicity of clove oil against *T. castaneum*. The *n*-hexane fraction of clove oil was 1.4 times more toxic than crude oil (non-fractionated) as shown by their compared LD_{95} values. Non-fractionated clove oil, in a concentration of 15% (equal to the dose of 0.75 ml/l fumigation chamber) only caused 82% mortality, while the same dose of the *n*-hexane fraction caused 100% mortality.

We also find that unfractionated clove essential oil is comprised of many diverse chemical components, such that subsidiary compounds will affect the performance of the primary active component by dilution or adulteration of the intended effect. Fractionation illustrates the difference in amount of *n*-hexane and ethyl acetate fractions obtained. This aligns with an assumption that most of the chemical compounds in clove oil are non-polar, and will bind to non-polar solvent (*n*-hexane) resulting in a greater amount of fraction than from the binding of the minority chemical compounds to semi-polar (ethyl acetate) and polar (methanol) solvents.

Clove oil contain 18 chemical compounds: 2-heptanone (0.04%), α-pinene (0.01%), *p*-cymene (<0.01%), limonene+1.8 cineole (0.01%), 2-heptyl acetate (0.04%), (E)-β-ocimene (0.33%), 2-nonanone (0.02%), linalool (0.01%), methyl salicylate (0.07%), *p*-llyl phenol (0.19%), eugenol (87.00%), α-copaene (0.10%), β-caryophyllene (3.56%), α-humulene (0.40%), Δ-cadinene

Table 2. Mortality of *Tribolium castaneum* treated with clove oil-based fumigant tablet

Treatments[a]	Mortality after fumigation (%) at…..days		
	3	5	7
Control	0 b	0 d	4 d
I	0 b	2 d	50 c
II	53 a	99 a	100 a
III	49 a	78 b	91 ab
IV	9 b	44 c	73 b

[a]Treatment with different composition of essential oils and naphthalene (essential oil:naphthalene), I= 3:0, II= 0:3, III= 1:1, IV: 2:1.

(0.04%), eugenyl acetate (8.01%), caryophyllene oxide (0.10%), and 2(12),6(13)-caryophyllen-dien-5-ol (0.02%) (Alma et al. 2007). The chemical components of the *n*-hexane fraction of clove oil that are toxic to *T. castaneum* and have general insecticidal effects are eugenol and beta-carryiophyllene. We found a single dominant compound in the *n*-hexane fraction of clove oil (eugenil acetate) but we did not find that compound in our GC-MS results. Eugenil acetate is known to be the dominant chemical compound in cloves generally, and its concentration determines the quality of clove oil (Alma et al. 2007). The content of eugenil acetate in clove oil ranges between 8.01–12.43% (Alma et al. 2007; Nassar et al. 2007; Prianto et al. 2013). Our results indicate nonetheless that the levels of this dominant chemical component in clove essential oil neither determined its toxicity level, nor increased its effectiveness.

Conversely, naphthalene—on its own or as an addition to other treatements-- significantly impacted *T. castaneum* mortality. This was illustrated by mortality results of 100% for all tablet treatments containing. Similar findings were reported by Damayanti et al. (2013), where mortality of *Rhyzopertha dominica* (Fabricius) treated with fumigant tablets containing a mixture of neem oil and naphthalene was slightly higher than when treated with neem oil only. Aside from the composition of the tablets, exposure time also affected the level of mortality of *T. castaneum*. The longer the exposure time applied caused the higher the mortality occurred.

CONCLUSIONS

Clove oil has potential for development as an alternative fumigant to phosphine or other commonly used pesticides, and fractionation of clove oil can increase its toxicity against *T. castaneum*. In our study, the LD_{50} and LD_{95} values of crude clove oil against. *T. castaneum* were 0.234 and 1.142 ml/l fumigation chamber, respectively. The *n*-hexane fraction of clove oil was 1.4 times more toxic than its crude one (non-fractionated) based on a comparison of their LC_{95} values. LD_{50} and LD_{95} values for the *n*-hexane fraction of clove oil against *T. castaneum* were 0.185 and 0.801 ml/l fumigation chamber, respectively. The primary components found in the *n*-hexane fraction of clove oil were eugenol and beta-caryophyllene. Clove oil tablets containing a mixture of clove oil and naphthalene (1:1) caused 91% mortality at 7 days' fumigation against *T. castaneum*.

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