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Essential oils from *Ellettariopsis slahmong* C. K. Lim and *Cinnamomum burmanii* [Nees & T. Nees] Bl. inhibit the colony growth of *Phytophthora palmivora* of cocoa

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ABSTRACT

Phytophthora palmivora fungal pathogen is the cause of cocoa's current slump of production in Indonesia. This has prompted the Government of Indonesia to initiate a rehabilitation by replanting 450 000 ha to boost national cocoa [*Theobroma cacao*] bean production to 1 million tons in 2013. Since 2006, the Province of West Sumatra has selected to support the program in the western part of Indonesia. As the result, the development of cocoa planting area in West Sumatra increase significantly less in a decade, from 25 000 ha in 2005 to 137 355 ha in 2013. However, its productivity constrained due to pests and diseases, mainly black pod disease caused by *Phytophthora palmivora*. Therefore, the average of cocoa yield in this province only 300-700kg / ha while its genetic potential is up to 2000 kg / ha. The purpose of this research was to study in vitro the fungicide effect of formulated essential oils of *Ellettariopsis slahmong* and *Cinnamomum burmanii* against *P. palmivora*. The method used was completely randomized design [CRD] in factorial. The first factor was the type of essential oil formulas: *Cinnamomum burmanii* litterfall leaf essential oil, wild Zingiberaceae *Ellettariopsis slahmong* essential oil, and a mixture of them both. The second factor was the level of concentration of essential oils starting from 250 ppm, 500 ppm, 750 ppm

and 1000 ppm per 100 mL of potato dextrose agar [PDA] media and control. Observation parameters were the diameter of colonies, colony growth velocity, biomass of colony and resistance test. The best result was the mixture treatment of essential oils of wild Zingiberaceae *Ellettariopsis slahmong* and *Cinnamomum burmanii* litterfall leaf by using concentration of 1000 ppm. The treatment successfully inhibited *P. palmivora* colony growth, miselium and hyfa. This result strongly indicated that the combination of these essential oils can be developed as a bio fungicide.

Keywords: Indonesia's cocoa bean production 1 million tons per year, *Phytophthora palmivora*, wild Zingiberaceae, litterfall leaf, Bio fungicide.

INTRODUCTION

The use of chemical pesticide in crop insect and disease control programmed around the world has resulted in damage to the environment, pest and pathogen resurgence, pest and pathogen resistance and lethal effects on non-target organisms. The negative effects which are mentioned above has forced scientist to search of alternate techniques for the management of controlling pests and pathogens by using biopesticide [1-17]. Biopesticide - derived from some parts of active ingredients of plants - as part of integrated pest management has been suggested worldwide as the most sustainable long-term solution [4, 17]. The biopesticide characteristics are biodegradable, specific target and good compatibility with the environment [17, 14].

The fungal pathogen *P. palmivora* is a major biological factor that limit cocoa *Theobroma cacao* L production worldwide up to 90% [3, 5, 7]. This pathogen cause cocoa black pod disease and limits annual cocoa yield in West Sumatra Indonesia by only 300-700 kg per ha [11] from its 2000 kg genetic potential yield per ha [13]. Though the province is set to be the center of the development of cocoa in the West Indonesia since 2006 and has achieved the planting area 137 000 ha in 2013 [18], from 25 000 ha in 2006 [13]. However, as in the situation centered cocoa development in Eastern Indonesia [Sulawesi] where the attack of pests and pathogens significantly decreased cacao production [19], in West Sumatra the situation worse due to mainly fungal pathogen *P. palmivora*, [20].

Traditional method of chemically controlling pathogens which commonly used by growers is expensive, has negative impact and ineffective [17, 14]. It has proven by high levels of *P. palmivora* attacks in Indonesia [11, 21-23]. Accelerate the distribution of these pathogens not only because it is less maximal application of the system of cultivation Good Agriculture Practices [GAP] for farmers [11, 24], but also due to the 6 – 9 months' rainy season in western part of Indonesia including West Sumatra [25] and ant *Iridomirmex cordatus* commonly found in plantation cocoa as a vector *P. palmivora* [26,9]. In Sulawesi, yield losses due to *P. palmivora* attack during the rainy season can reach 60% [27]. From field observations since 2008 to 2016 in West Sumatra, obtained data is that none of the cocoa plantations free from *P. palmivora* attack, including at a 400ha private estate in Agam District [11, 23]. This study aimed to observe the biopesticide effects of essential oils of wild Zingiberaceae *Elettariopsis slahmong* C. K. Lim and litterfall leaf of *Cinnamomum burmannii* [Nees & T. Nees] Bl against *P. palmivora* of cocoa.

Elettariopsis genus are reported to contain anti-microbial [6, 28, 29]. According to Nasir and Nurmansyah [22] and Nasir [24], essential oil of *E. slahmong* with a dose of 1000 ppm managed to control the antrachnose disease caused by *Colletotrichum*

gloeosporioides on dragon fruit up to 100%. While Nurmansyah [25,27] reported that a dose of 500 ppm essential oil of *C. burmannii* successfully controlled *Sclerotium rolfsii*, *Fusarium* sp and *Phytophthora capsici* in the range of 88.67 - 100%. As a source for biopesticide, both plants are widely available in West Sumatra Indonesia.

RESEARCH METHODS

This research was conducted from March to September 2016 at the Laing Research Station of Bogor Research Institute for Spice and Medicinal Plants in the District of Solok, and in the Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, both are in the Province of West Sumatra. The design used is Completely Randomize Design in factorial with 12 treatments and 3 replications. The treatment is three kinds of essential oils [A1: essential oil of leaf litterfall *C. burmannii*, A2: wild Zingiberaceae essential oil *E. slahmong*, A3: a mixture of essential oil *C. burmannii* + essential oil *E. slahmong* and four doses per treatment essential oils [B1: 250 ppm, B2: 500 ppm, B3: 750 ppm, B4: 1000 ppm] in 100 mL PDA each. Four types of activity were recorded: [1] the inhibition of essential oils on the growth of colonies of *P. palmivora*. Growth inhibition determined by the presence of an inhibition zone; [2] The effect of the concentration and type of essential oil in inhibiting the growth of fungal colonies; [3] Biomass colonies of *P. palmivora* [4] Pathogen resistance test.

Phytophthora isolates

Phytophthora palmivora isolate was obtained from infected cocoa fruit was used in this study and was kindly provided by the Indonesian Coffee and Cocoa Research Institute in Jember, East Java Indonesia. The isolate was routinely grown on PDA at $25 \pm 1^\circ\text{C}$ [29] and used for the following works in this study. In all stages of this research, pathogen inoculum comes from single zoospore purified.

Essential oils

Formulated 25% concentration of each essential oils of *E. slahmong* and *C. burmannii* were obtained from Biology Department Andalas University and Laing Research Station in Solok. The essential oils were tested in vitro against *P. palmivora*. Bioassay protocols were carried out based on Perez *et al* [29] which was modified. To obtain inoculum, a plug of 2.5 mm diameter *P. palmivora* colony was transferred onto a PDA medium and incubated in the dark at 28°C , until the mycelium was approximately 10 mm from the edge of the Petri dish. This culture then was used for the following bioassays work to find out biopesticide effect of the tested essential oils.

Preparation of media

The instant Potato Dextosa Agar [PDA] was used in this study. While Potato Dextrose Broth [PDB] prepared as follows: the potatoes peeled and cut into small pieces. Weighed as much as 20 g, heated to boiling, taken the extract then added 1 g of dextrose up to 100 ml and stirred until homogeneous. Sterilized by autoclave at a temperature of 121°C for 15 minutes.

The ability of inhibition of essential oils against colonies growth of P. palmivora

In vitro bioassay was conducted to test the inhibition ability of essential oils to pathogen by growing *P. palmivora* [source culture] on PDA that had been treated. Testing was done by mixing each formulated essential oil according to treatment into 100 mL of PDA that is still melting [45°C] and stirred until homogeneous. Media that already contains the essential oil was poured into sterile petridish and allowed to harden. Then transplanted 2.5 mm diameter pieces of isolate of *P. palmivora* [by using sterilize cork borer] in the middle of the media and incubated at 28°C for 7 days and put in a dark place [31]. As a control, isolate of *P. palmivora* was grown on PDA without essential oil. The observation was done by measuring the diameter growth of isolates of *P. palmivora*. Observation was stopped when the fungus in the control treatment has grown to meet the edge of media in 9 cm petridish. To test the inhibitory effect of formulated *E. slahmong* and *C. burmanii* against *P. palmivora* colony growth was calculated according to Awang *et al* [2]:

$$I = \frac{C-T}{C} \times 100\%$$

I = Inhibition of colony growth

C = diameter of fungal colonies in the control treatment

T = diameter of fungal colonies on the treatment treated

The ability of essential oil in inhibiting the colonies growth velocity of P. palmivora

Testing is done by mixing each treatment essential oil in 100 mL of PDA that is still melting [45°C], then stirred until homogeneous. Media containing formulated essential oils that have been poured into sterile petridish and allowed to harden. Then transplanted isolates [culture source] *P. palmivora* use corkborer diameter of 2.5 mm in the middle of the media that have been treated. *P. palmivora* cultures incubated at 28 °C for 7 days. As a control treatment, isolate of *P. palmivora* was grown on PDA were without essential oil. Data were collected for diameter growth of isolates of *P. palmivora* and stopped when *P. palmivora* in the control treatment has met the edge of petridish [9 cm] or at least 10 mm from the edge.

Biomass colonies of P. palmivora

Tests using 5mL medium of PDB which was inserted into each 25-ml test tube and sterilized in autoclave. When the liquid media was still in 45°C, the formulated essential oils was added according to treatments, homogenized and hardened. Then 2.5 mm isolates [source culture] *P. palmivora* previously grown for 7 days in medium PDA was transplanted and incubated in an incubator at a temperature of 28°C for 7 days. Furthermore, colonies of mold growing *P. phytophthora* was separated from the media, then dried in an oven at 60°C for 24 hours and weighed [32].

Pathogen resistance test

Resistance tests conducted by regrown *P. palmivora* isolate derived from the treatment that have the highest inhibitory effect. Isolates were grown for 7 days at a temperature of 28°C and placed in the dark [33]. An assessment of the level of resistance of pathogens carried by observing the growth of *P. palmivora*, whether grow back after being transferred to PDA medium. If re-growth occurs, it means the treatment is merely fungistatic. If growth does not occur again, then the essential oil has a potential to be a bio fungicide.

Data analysis

Data obtained from observations of inhibition of essential oil to the colony *P. palmivora* statistically tested. If the test Analysis of Variance [ANOVA] F arithmetic value higher than F table at the 5% significance level, it will be followed by a further test of Duncan's Multiple Range Test [DNMRT].

RESULTS AND DISCUSSION***Inhibition of essential oils on the growth of colonies P. palmivora***

According to Chairgulprasert *et al* [6], antimicrobial capability of essential oil of *E. slahmong* is very high. It has terpenoid compounds that disrupt protein synthesis and inhibited cell division and multiplication. While Nurmansyah [26] found that volatile compounds produced from oil leaves, twigs, and bark of *C. burmanii* able to suppress the growth of *P. capsii* in vitro. In this study, either formulated essential oil of *E. slahmong* or *C. burmanii* able to suppress the growth of *P. palmivora*, although the two are not significantly different. But when formulated *E. slahmong* essential oil was mixtured with essential oils [34], of formulated *C. burmanii*, inhibitory effect on the growth of colonies of *P. palmivora* increased and significantly different with a single treatment and control [Table 1]. These results are consistent with the statement of Wong *et al* [35] that the antimicrobial effect of essential oils will be more effective, when the use of some types of essential oils are mixed, as some have the effect of synergism.

Table-1: Effect of essential oils on the inhibition of colonies growth (%) of fungal pathogen *P. palmivora* after 6 days of inoculation.

No.	Treatment	Inhibition growth (%)
1.	<i>E. slahmong</i>	53.65b
2.	<i>C. burmanii</i>	53.94b
3.	Mixture	72.40a
4.	Control	0 .00c
CV: 5.118 The numbers followed by the same small letter in each column are not significantly different at 5% test DNMRT		

In this study also found that increasing the concentration of essential oils is directly proportional to the increase in the percentage of its inhibition rate, where any increase in the concentration of 250 ppm, an increase in the percentage of inhibition of $\geq 5\%$ [Table 2]. According to Harsari [12], the increasing concentration of essential oils affect its ability to inhibit the growth of fungal pathogen.

Table 2. Effect of the concentration levels on the inhibition of colony growth (%) of the fungal pathogen *P. palmivora* after 6 days of inoculation

No.	Treatment (ppm)	Inhibition ability (%)
1.	250	33.16d
2.	500	60.08c
3.	750	70.64b
4.	1000	76.12a
5.	Control	0.00e

CV: 5.118: The numbers followed by the same small letter in each column are not significantly different at 5% test DNMR

Essential oils of litterfall leaf *C. burmanii* at a concentration level of 0.02% has been able to suppress the growth of *Sclerotium rolfsii* of pepper and peanuts at 88.67 to 99.81%. At the concentration level of 0.05%, it has been showing 100% inhibition of the growth of *S. rolfsii* [27]. Furthermore, volatile compounds of essential oils *E. slahmong* at a dose of 0,5ml / petri able to inhibit the growth of colonies of *C. gloesporoides* [22]. In this study, the important role of concentration and mixture was found on the mixture of formulated essential oil *E. slahmong* with formulated essential oil *C. burmanii* [A3B4] at concentration of 1000 ppm [Table 3]. This treatment resulted the highest inhibition rate on the growth of colonies *P. palmivora* thus was 85.86%.

Table-3: Effect of essential oils and levels of concentration on the inhibition of colonies growth (%) *P. palmivora* after 6 days of inoculation.

No	Treatment (ppm)	Inhibition rate (%)
1	A1B1 (250 <i>C. burmanii</i>)	34.76h
2	A1B2 (500 <i>C. burmanii</i>)	52.53f
3	A1B3 (750 <i>C. burmanii</i>)	60.33e
4	A1B4 (1000 <i>C. burmanii</i>)	66.99de
5	A2B1 (250 <i>E. slahmong</i>)	19.59i
6	A2B2 (500 <i>E. slahmong</i>)	50.36fg

7	A2B3 (750 E. slahmong)	70.33cd
8	A2B4 (1000 E. slahmong).	75.50bc
9	A3B1(250 C. buramanii+250 E. slahmong)	45.13g
10	A3B2(500 C. buramanii+500 E. slahmong)	77.36bc
11	A3B3(750 C. buramanii+750 E. slahmong)	81.26ab
12	A3B4(1000 C. buramanii+1000 E. slahmong)	85.86a
13	Control	0.00j
CV: 5.118: The numbers followed by the same small letter in each column are not significantly different at 5% test DN MRT.		

According to Knobloch *et al* [15] and Kumar *et al* [16], terpenoids, decanoic acid and esters derived from essential oils can accumulate globules of fat in the cytoplasm, inhibiting the growth of conidia and mycelium and shortening the tips of the hyphae. Essential oil of *E. slahmong* contains chemical components mentioned above [22-24] which has been implicated in this study by inhibiting the growth of spores and hyphae of *P. palmivora* [Figures 1 and 5]. Meanwhile Wang *et al* [34] reported that the main component of essential oil of *C. burmannii* is transcinamaldehyd [60.72%], eugenol [17.62%] and coumarin [13.39%], which has proved capable of controlling *Phytophthora capsici* causes stem rot disease on pepper [27]. Volatile compounds produced from oil leaves, twigs, and bark of *C. burmannii* reportedly also able to suppress the growth of plant pathogenic fungi in vitro [26]. In this study, the accumulation of both essential oils content is able to suppress the growth of colonies of *P. palmivora* [Table 1, 3 and Fig 1].



Figure-1: Comparison of colony diameter of *P. palmivora* in various essential oil treatments after 6 days. The best treatment is the mixtured treatment of 1000ppm formulated *E. slahmong* essential oil with formulated *C. burmanii* essential oils (A3B4 bottom right).

The effect of formulated essential oils on the colony growth velocity of P. palmivora

Observations were made one day after the inoculum of *P. palmivora* [source culture] transplanted in PDA medium according to treatment and observed daily until day six. All treatments of essential oils, either single or mixed suppress the growth of colonies of *P. palmivora* [Figure 2]. This pressure varies, but the mixture treatment of *E. slahmong* formulated essential oil and *C burmanii* formulated essential oil at the concentration of 1000 ppm [A3B4], pressing fastest the colony growth and suppress it until the end of the observation except on day three. Harni *et al* [12] reported that *P. palmivora* isolates which were inoculated on cacao fruit without treatment, causing symptoms blackish brown spots starting on day 2 and within six days spotting size widened and showed a clear boundary between the symptomatic disease and healthy fruit. Figure 2 is the phenomenon of colony growth until the sixth day of treatment with various essential oils.

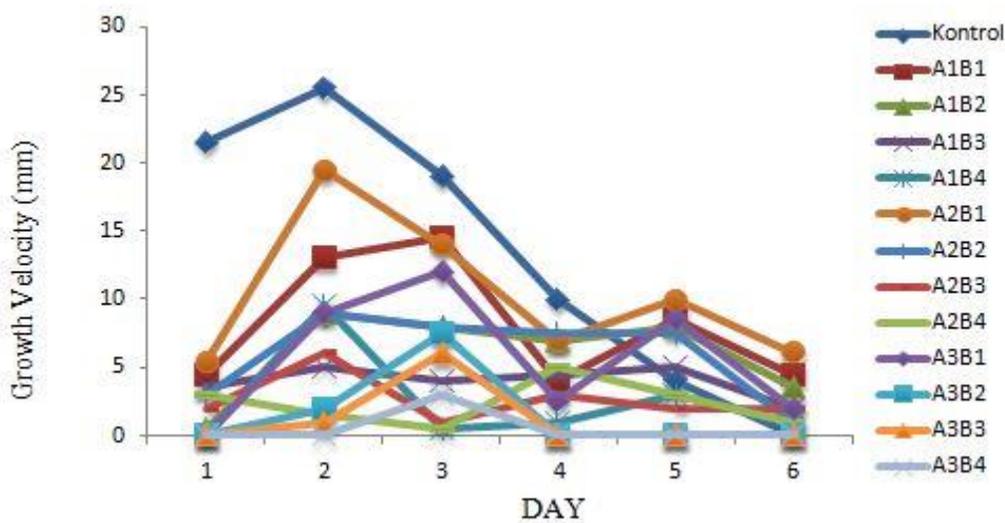


Figure-2: Fluctuations in the colonies growth velocity of *P. palmivora* after a day of application of essential oils. The combination of formulated essential oil *E. slahmong* and formulated essential oil *C burmanii* at the concentration of 1000 ppm (A3B4) consistently suppressed colony growth, after three days until end of observation at day sixth.

Biomass of P. palmivora

Weight of colonies of *P. palmivora* in each treatment was not much different as seen in Figure 3 whereas some treatments have a weight close to zero.

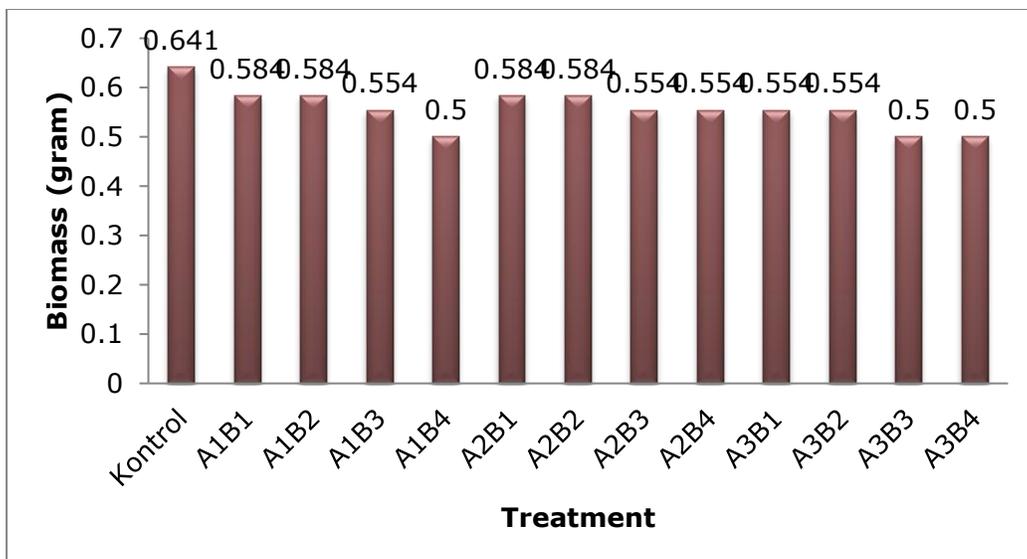


Figure-3: The difference in weight of biomass colonies of *P. palmivora* due to the treatments of formulated essential oils of *E. slahmong* and *C. burmanii* at 6 days of incubation.

In term of biomass, the treatment of a mixture of 1000 ppm *E. slahmong* formulated essential oil with 1000 ppm *C. burmanii* formulated essential oil [A3B4] gave the best results as seen in Figure 4.



Figure-4: Effect of treatment of essential oils to biomass colonies of *P. palmivora*; (a). colony grown on media PDA without essential oil treatments /control (b). media PDA amended with 1000 ppm of the mixture *E. slahmong* and *C. burmanii* (treatment A3B4), there was no colony growth (c). Colony.

Chemical constituents, anti-microbial properties, concentration of the treatment and treatment mixture looks very influential on biomass in this study [Table 1, Fig 2,3,4]. Also, efficacy of essential oil of *E. slahmong* and *C. burmanii* in controlling *Phytophthora palmivora* in this study are supported by previous studies [22-27, 35].

Resistance test pathogen

Although Figure 3 showed that there are three treatments that produce the same biomass weight of 0.5 g, but the most rapid and stable in suppressing colony *P. palmivora* was caused by the mixture of formulated essential oils *E. slahmong* with *C. burmanii* at a dose of 1000 ppm [A3B4] [Figure 2], similar to other results in this study. The efficacy of treatment was seen in the inhibition of the growth of colonies [Table 3] as well as the morphology and microscopic damage to the colony and hyphae [Figure 5].

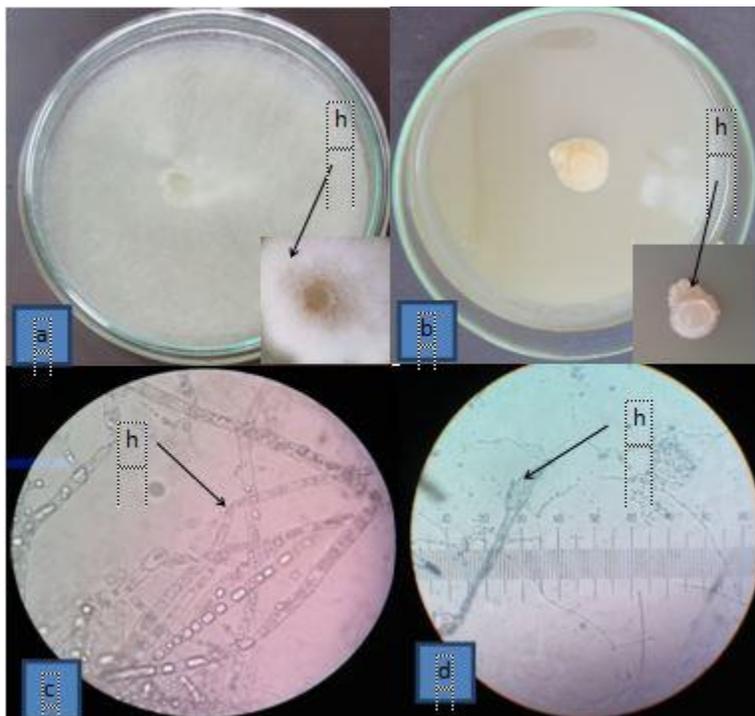


Figure-5: Comparison of normal macroscopic, microscopic and damaged hyphae caused by the treatment (a). Macroscopically normal hyphae; (b). Macroscopic damage hyphae (c). Microscopic normal hyphae (d). Microscopic damage hyphae which causes abnormal branching and undeveloped hyphae (h), Hyphae.

There are two kinds of resistance in fungi and have different causes [7]. The first is resistance physiological adaptation, are generally unstable and will recover if exposure to the fungicide is stopped. While resistance of genetic mutation is generally stable and intractable [8]. Further research is needed to determine the nature of resistance from the results of this study. The damage caused by the antimicrobial component can be fungicidal [kills fungi] and fungistatic [temporarily stop the growth of fungi]. In this study, there was no colony of *P. palmivora* developed up to 7 days after regrowth. Although in this study suspected mixture of formulated essential oils of *E. slahmong* and *C. burmanii* at a dose of 1000 ppm [A3B4] kills the fungus. but necessary to conclude a further test which is currently underway.

CONCLUSION

The mixture of formulated essential oils *E. slahmong* and *C. burmanii* with a concentration of 1000 ppm gave the best results in inhibiting the growth of colonies of *P. palmivora* and it was indicated three days after inoculation.

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