International Journal of Agricultural Technology 2021Vol. 17(6):2363-2374
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

In vitro effect of *Callistemon viminalis* and *Melaleuca cajuputi* ethanolic extracts as botanical fungicide and insecticide

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Somnuek, S., Thipmanee, K. and Jaenaksorn, T. (2021). *In vitro* effect of *Callistemon viminalis* and *Melaleuca cajuputi* ethanolic extracts as botanical fungicide and insecticide. International Journal of Agricultural Technology 17(6):2363-2374.

Abstract Effect of ethanolic extracts of *Callistemon viminalis* (EECV) and *Melaleuca cajuputi* (EEMC) leaves was evaluated in laboratory against plant pathogenic fungus (*Alternaria* sp.) and plant insect pests (*Aphis craccivora* and *Phenacoccus manihoti*). Regarding the study on botanical fungicide, the combined formulas of EECV and EEMC (5000 ppm) in ratios of 100:0, 75:25, 50:50, 25:75 and 0:100 were tested on mycelial growth and spore germination of tested fungus. The results revealed that all tested formulas of plant extracts gave significant inhibition on mycelial growth and spore germination in the ranges of 27.5-92.7% and 75-100%, respectively. However, the EEMC itself was unexpectedly found highly effective than the combined formulas. In the case of insecticidal activity, the preliminary experiment of EECV and EEMC (2000, 10000 and 20000 ppm) was performed on aphid and mealybug. The result presented that all concentrations of EECV and EEMC significantly showed the mortality effect on aphid and mealybug about 5-100%. In addition, the EECV and EEMC at 2000 and 10000 ppm showed a repellency of about 5-55%. Then, both plant extracts at 2500, 5000, 10000 and 20000 ppm concentrations were tested to those insects. The 100% mortality of aphid and mealybug was found in the highest concentration of EECV and EEMC while 2500-10000 ppm concentrations showed in the range of 38-98%. The 100% repellency capacity was observed at 10000 ppm concentration. Therefore, two Myrtaceae ethanolic extracts are promising to be used as a natural pesticide in controlling *Alternaria* and insect pests in laboratory.

Keywords: Myrtaceae ethanolic extract, Botanical fungicide, Botanical insecticide, *Callistemon viminalis*, *Melaleuca cajuputi*

Introduction

Vegetables are highly susceptible to insect pests and diseases. Therefore, pests and diseases are considered as a major limiting factor in the commercial vegetable production (Hoffmann *et al*., 2004). Several insect pests including aphids, mealybugs, beetles and cutworms cause significant damage (up to 60% yield losses) to vegetable crops (Shankar and Raju, 2012). *Alternaria* spp. is one of the most widespread genera in the world that causes pre- and post-

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harvest damage to agricultural products including vegetables (Lopez and Cabral 1999). For instance, *Alternaria* infection of broccoli and cauliflower heads can lead to complete deterioration of the heads and results in total loss of marketability (Singh *et al*., 2015). To combat such a various insect pests and diseases, most growers rely almost exclusively on chemical synthetic insecticides and fungicides but without satisfactory control result. On the other hand, the disadvantages to widespread synthetic pesticide use are significant. Chemical pesticides in agriculture have also imposed a serious negative impact on the environment such as contamination of soils and groundwater and contamination of crop products with harmful chemical residues (Bahar *et al*., 2007). Therefore, long-term sustainable management can help growers decrease their reliance on chemical insecticides and fungicides. At present, public awareness of pesticides residues on agricultural produces as well as on environmental problems has stimulated research efforts using natural products to reduce the impacts of pests and diseases on agricultural systems. A renewed interest in the use of botanical pesticides for crop protection without other adverse effects on the host (such as reduced yield) has increased markedly in the last 10 years (Wurms, 2020).

Callistemon viminalis (bottle brush tree) and *Melaleuca cajuputi* (cajeput), belong to the family Myrtaceae, were used in traditional and folk medicine (Ko Ko *et al*., 2009; Ahmad and Athar, 2017; Mohamed *et al*., 2017). Moreover, the extracts and essential oil from the plants in this family have been reported to have toxic effect on microorganisms and insect pests (Ko Ko *et al*., 2009; Visheentha *et al*., 2018; Bakar *et al*., 2019). However, only a few and limited studies of the extract of *C. viminalis* and *M. cajuputi* as botanical fungicide and insecticide was noted.

Therefore, this research was conducted to determine the effect of ethanolic extracts from *C. viminalis* and *M. cajuputi* as botanical pesticides to control *Alternaria* sp. and insect pests (aphid and mealybug) in a laboratory condition.

Materials and methods

Plant ethanolic extract preparation

Callistemon viminalis (Bottle brush tree) leaves were collected from the area in King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok province while *Melaleuca cajuputi* (Cajaput tree) leaves were collected in KMITL Prince of Chumphon Campus, Chumphon province, Thailand. Both plant leaves were washed with tap water and dried in the open air. The leaves were then dried in hot air oven at 50℃. Blender was used to grind the dried samples. The Soxhlet method was used to extract the plant leaves. The ethanolic solvent of obtained crude extracts were removed by rotary evaporator. Then the ethanolic crude extract of *Callistemon viminalis* (EECV) and *Melaleuca cajuputi* (EEMC) were kept in refrigerator at 4 ℃ for further study.

Sample preparation

Plant pathogen

Alternaria sp. causing leaf spot disease of lettuce was used in this study. The fugus was maintained in potato dextrose agar at 7 days old. Then spore suspension of *Alternaria* sp. was prepared and adjusted to 10^6 spores/ml for this study.

Insect

The tested insects as aphid and mealybug were collected initially from agricultural farm at Faculty of Agricultural Technology, KMITL, Bangkok. Both aphid and mealybug were reared and multiplied on alternate host in greenhouse further experiment.

Effect of plant ethanolic extracts on Alternaria sp.

Mycelial growth test

Effect of antifungal activity of EECV and EEMC on the mycelial growth of *Alternaria* sp. was carried out by poisoned food technique. The EECV and EEMC alone (100:0 and 0:100) including the combination of EECV and EEMC in ratios of 75:25, 50:50 and 25:75 were used in this experiment. The molten PDA was mixed with each formula of the tested plant extracts at a 5000 ppm concentration. Then the PDA with plant extract was poured into Petri dish (5 cm diameter). A mycelial disc of 5 mm diameter was inoculated on the center of the prepared plate. A completely randomized design (CRD) with 5 replications was used in the experiment. Ten percent of dimethyl sulfoxide (DMSO) without plant extract was used as a control treatment. After that, the tested plates were incubated at 25 ℃. The diameters of the colony growth in control and plant extract treatment were measured at 1, 3, 5 and 7 day and the antifungal effect was calculated by the following formula:

$$\text{Antifungal activity (\%)} = (Dc-Dt)/Dc \times 100$$

where Dc was the diameter of colony growth in control plate.

Dt was the diameter of colony growth in the plate containing tested extract.

Spore germination test

To evaluate the antifungal activity of EECV and EEMC on *Alternaria* sp. by spore germination test, the experiment was carried out in CRD with 5 replications. The experimental treatments were EECV mixed with EEMC in the ratio of 0:100, 25:75, 50:50, 75:25 and 100:0 at 2500 ppm. One ml of prepared spore suspension (10^6 spores/ml) was added with one ml of the tested plant extract at desired final concentration. Ten percent of DMSO without plant extract was used as control treatment. The spore germination was observed under light microscope at 12, 24, 36, 48, 60 and 72 h. The inhibitory effect was compared with the control treatment.

Effect of plant ethanolic extracts on insect pests

Preliminary test of plant extracts on insect

To assess the effect of EECV and EEMC on aphids and mealybugs, the lowest, medium and highest concentrations (2000, 10000 and 20000 ppm) of the tested plant extracts were used in this experiment. Each concentration of the plant extract was filled to filter paper of 5 cm in diameter and placed into Petri disc (5 cm in diameter). Then twenty tested insects were transferred on the prepared filter paper. The experiment was designed in CRD with 3 replications. The 10% tween20 without plant extract was used as a control treatment. The mortality effect was observed at 6, 12 and 24 h and calculated followed by mortality effect (%) = ((NC-NT)/NC) × 100 where NC was the number of insects in the control treatment while NT was the mortality number of insects in plant extract treatment. In addition, the preliminary repellency of the tested plant extracts was determined at the same time.

Mortality effect of plant extracts on insect pests

The mortality effects of EECV and EEMC at 2500, 5000, 10000 and 20000 ppm concentrations against aphids and mealybugs were carried out by paper contact technique. The experimental method and design were tested in the same way on the preliminary test of the insect. The nine cm in diameter of Petri dishes were used in this experiment. In addition, probit analysis of lethal concentration fifty (LC50) value was evaluated.

Repellent effect of plant extracts on insect pests

The repellent effect of EECV and EEMC on aphids and mealybugs were performed using the different low concentrations (1250, 2500, 5000 and 10000 ppm) as compared with mortality test. Half of the filter paper (9 cm in diameter) was added by one mL of the tested plant extract whereas, the other half filter paper was filled with 10% tween20 (control). Both the treated half and control half of paper filters were placed in a plate of 9 cm in diameter. Then twenty the tested insects were transferred to the center of the prepared filter paper. The CRD with 5 replications was used in the experiment. The repellent activity was observed at 12 and 24 h. The repellent percentage (RP) was calculated using the formula RP = ((NC-NP)/(NC+NP)) ×100 where NC was the number of tested insects on the control half and NP was the number of insects on the plant extract half.

Results

Effect of plant ethanolic extracts on Alternaria sp.

Effects of ethanolic crude extracts from *Callistemon viminalis* (EECV) and *Melaleuca cajuputi* (EEMC) leaf on mycelial growth and spore germination of *Alternaria* sp. were conducted. The tested formulations were the mixture of both EECV and EEMC in the ratios of 100:0, 75:25, 50:50, 25:75 and 0:100 at 5000 ppm concentration. The result showed that at 3 days after incubation (DAI), colony in control plate was about 50% whereas only slight mycelial growth was detected on the treatment plate. At 7 DAI, control plate reached 100% and inhibitory effects of the extracts revealed significantly about 28-93% (Figure 1 and 2). The stronger inhibitory effect was noted in EEMC alone (EECV+EEMC 0:100) about 93%. In the case of mixture extracts, three ratios (75:25, 50:50 and 25:75) significantly revealed the inhibition effect on the tested fungal mycelial growth in the ranges of 42-82%. The EECV+EEMC (25:75) presented the more significanct inhibitory effect than other mixture extracts (EECV+EEMC 75:25 and 50:50). While EECV alone showed a lower inhibition effect of about 28%. Regarding the spore germination test at 24 h after treatment, the spore germination in the control was about 24% while no-spore germination was found in the plant extract treatments. At 72 h, all tested formulas presented strong inhibition effect (about 77-100%) which were higher than in the mycelial growth test (Figure 1). the EEMC alone and EECV+EEMC 25:75 gave the highest inhibition on spore germination of the tested fungus about 98-100% followed by EECV+EEMC 50:50, 75:25 and EECV alone showed the effect about 91, 84 and 91%, respectively. However, the EEMC itself was unexpectedly found highly effective than the combined formulation (Figure 1). Moreover, abnormalities of spores such as swelling and lysis were detected after treating with the tested plant extract (Figure 2).

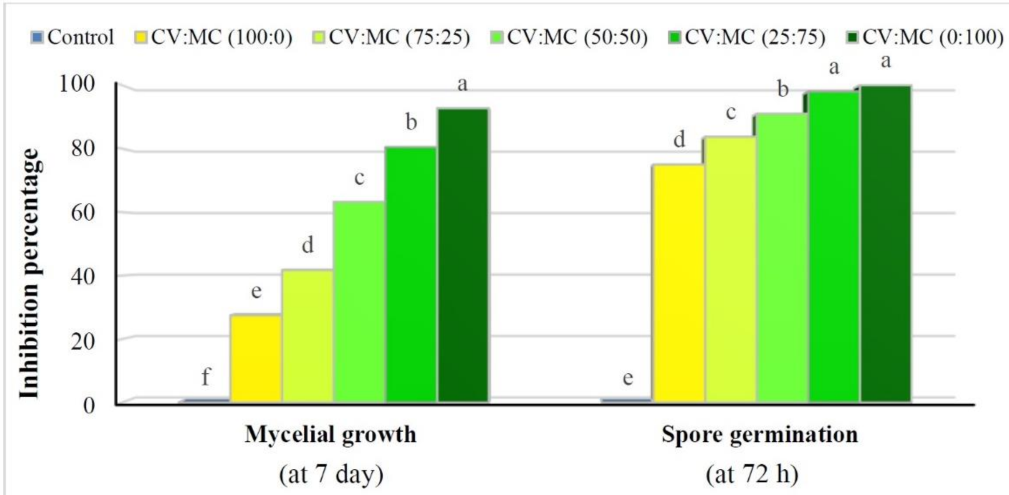

Figure 1. Inhibition effect of ethanolic extract from *C. viminalis* (EECV) and *M. cajuputi* (EEMC) on *Alternaria* sp. mycelial growth (at 7 day after incubation) and spore germination test (at 72 h)

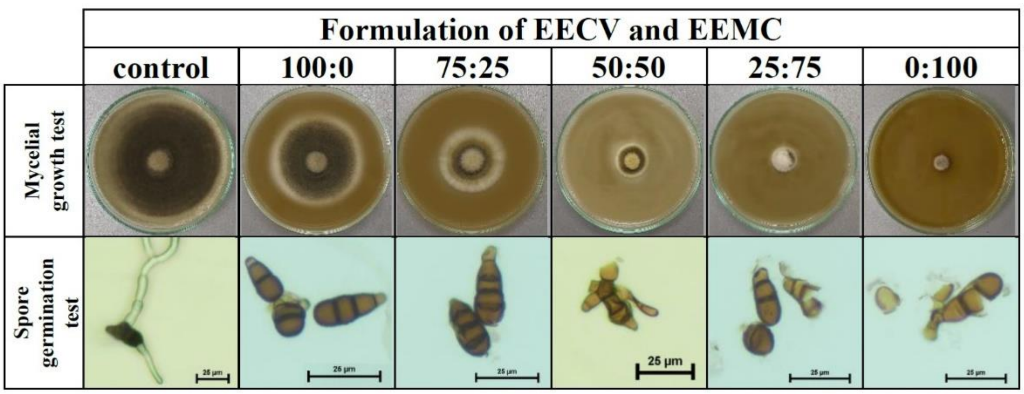

Figure 2. The characteristics of colony (at 7 day after incubation) and abnormal spore (at 72 h) of *Alternaria* sp. after being treated with plant extract formulation of EECV and EEMC : black bar = 25 µm

Effect of plant ethanolic extracts on insect pests

Preliminary test of plant extracts on insect

Effects of ethanolic crude extracts from *C. viminali* and *M. cajuputi* leaf were determined on aphid and mealybug. The result revealed that all concentrations of both tested plant extracts showed a wide range mortality effect on both tested insects. The highest mortality effect was noted at the highest concentration (20,000 ppm) followed by 10,000 and 2,000 ppm. The highest concentration (20,000 ppm) of EECV showed mortality effect on both tested insects about 80-90% while EEMC showed the effect about 100% at 24 h. The lowest concentration (2,000 ppm) of both plant extracts showed slightly the effect on the insect about 21-33%. However, EEMC showed effect more than EECV (Figure 3).

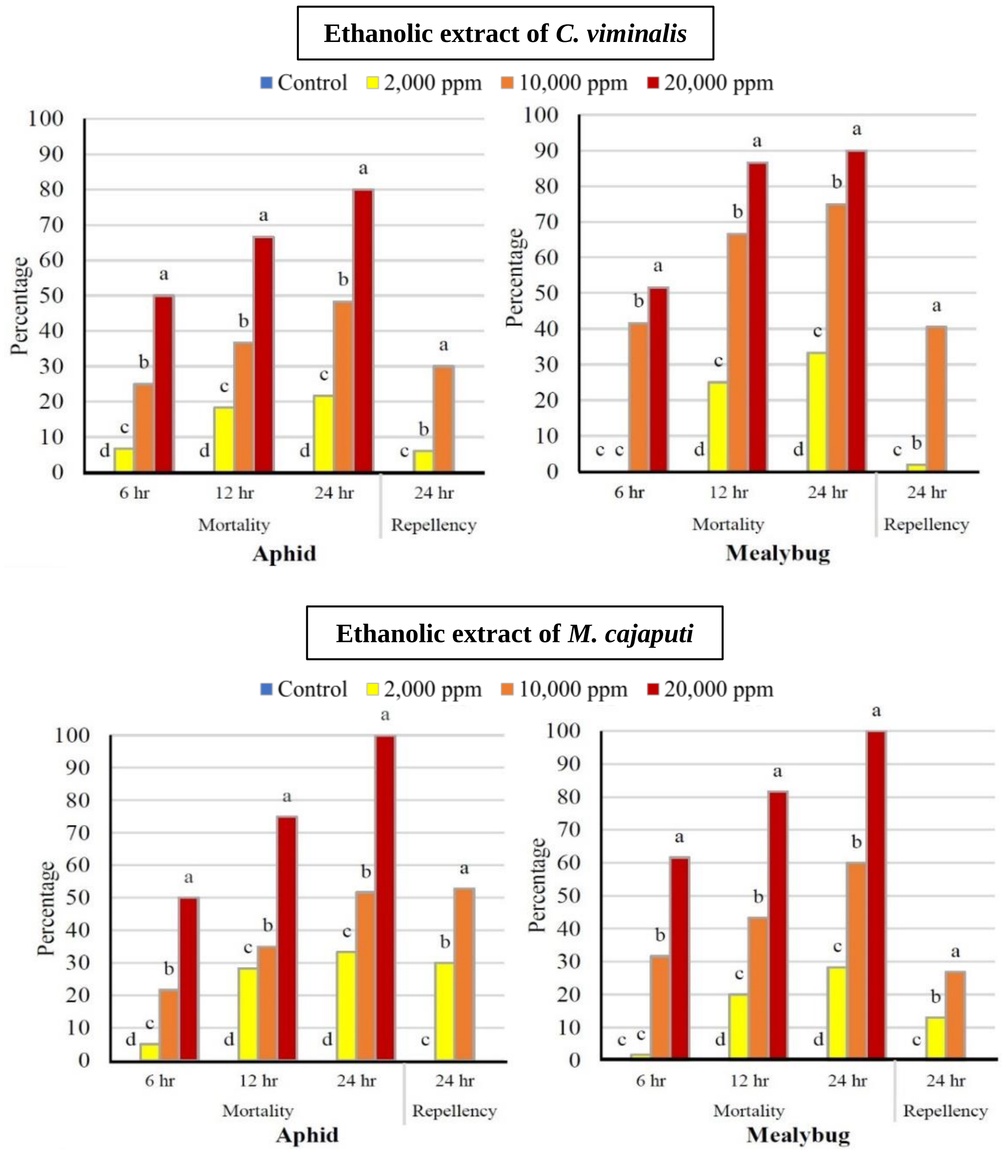

Figure 3. Mortality and repellency percentage of aphid and mealybug caused by ethanolic extract of *C. viminalis* and *M. cajaputi* using paper contact method

In addition, the result obtained from the repellent effect test in the medium and low concentration of both plant extract. The medium concentration (10,000 ppm) showed the repellent effect more than that of the lower concentration (2,000 ppm). The EECV (10,000 ppm) showed the higher repellency about 30 and 40% (aphid and mealybug respectively) while The EEMC showed the effect on aphid (52%) rather than mealybug (26%). The low concentration of both plant extract presented slightly the effect on both tested insects about 2-30%. The repellent effect fast occurred on the medium concentration more than the low concentration (Figure 3).

Table 1. Percentage and LC_{50} of aphid and mealybug caused by ethanolic extract of *Callistemon viminalis* (EECV) and *Melaleuca cajuputi* (EEMC) using paper contact method

Tested extract		**Mortality (%)**						**LC_{50} (ppm)**	
		Aphid (Ap)			**Mealybug (Mb)**			**Ap**	**Mb**
		12 h	**24 h**	**36 h**	**12 h**	**24 h**	**36 h**		
EECV	Control	0e	0e	0e	0e	0e	0e	2541	3801.6
	2500 ppm	5d	27d	43d	6d	28d	38d		
	5000 ppm	22c	37c	66c	20c	38c	64c		
	10000 ppm	30b	56b	87b	65b	77b	96b		
	20000 ppm	54a	75a	100a	73a	87a	100a		
EEMC	Control	0e	0d	0e	0e	0e	0d	2671	2981.2
	2500 ppm	7d	35c	49d	8d	33d	46c		
	5000 ppm	28c	42c	72c	22c	40c	60b		
	10000 ppm	46b	69b	83b	55b	72b	98a		
	20000 ppm	86a	99a	100a	81a	95a	100a		

Values are means of three replicates. Values in each column within each plant extract followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).
EECV = Ethanolic extract of *Callistemon viminalis*; EEMC = Ethanolic extract of *Melaleuca cajuputi*

Mortality effect of plant extracts on insect pests

Evaluation of the mortality effects of EECV and EEMC at different concentrations (2500, 5000, 10000 and 20000 ppm) on aphid and mealybug nymphs by using paper contact method demonstrated that all tested concentrations of both EECV and EEMC gave the mortality effect on tested insects. Among all tested concentrations, the highest concentration (20000 ppm) significantly presented the highest mortality of both aphid and mealybug nymph. In this regard, 12 and 24 h after treatments, the EECV (20000 ppm) presented the mortality percentage of mealybugs with more than that of aphids by the ranges of 73-87 and 54-75%, respectively. While the EEMC (20000 ppm) gave the higher mortality result on aphids more than mealybug with the ranges of 86-99 and 81-95%, respectively (Table 1). At the end of the test (36 h after the treatment), both EECV and EEMC at the highest concentration presented 100% mortality on two tested insects. Regarding LC50, EECV showed the concentration value on aphids and mealybugs about 2541 and 3801 ppm, respectively while EEMC showed the value about 2671 and 2981 ppm, respectively. However, when we considered LC50, the tested aphids showed a weaker response to the plant extracts than mealybug (Table 1).

Repellent effect of plant extracts on insect pests

The repellent effects of the different concentrations (1250, 2500, 5000 and 10000 ppm) of EECV and EEMC on aphid and mealybug nymphs were carried out. The results indicated that all tested concentrations of EECV and EEMC repelled the tested aphid and mealybug nymph with a wide range repellency, the lower to medium concentrations (1250-5000 ppm) of both EECV and EEMC gave the moderate repellency of both tested insects in the ranges of 14-62%. Moreover, the highest concentration of tested plant extracts showed the best repellent activity on the insects about 90-100% when compared to other treatments (Table 2). However, EEMC exhibited repellency on the tested insects better than the EECV.

Table 2. Repellency percentage of ethanolic extract of *C. viminalis* (EECV) and *M. cajuputi* (EEMC) against aphid and mealybug using paper contact method

Tested extract		Repellency (%)			
		Aphid		Mealybug	
		12 h	24 h	12 h	24 h
EECV	Control	0d	0d	0d	0d
	1250 ppm	0d	16c	0d	14c
	2500 ppm	22c	54b	14c	44b
	5000 ppm	48b	56b	28b	50b
	10000 ppm	88a	90a	86a	86a
EEMC	Control	0d	0d	0c	0e
	1250 ppm	6cd	24c	8c	22d
	2500 ppm	16c	58b	10c	28c
	5000 ppm	44b	62b	32b	56b
	10000 ppm	100a	100a	100a	100a

Values are means of three replicates. Values in each column within each plant extract followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).
EECV = Ethanolic extract of *Callistemon viminalis*; EEMC = Ethanolic extract of *Melaleuca cajuputi*

Discussion

Effect of crude ethanolic extracts from *Callistemon viminalis* (EECV) and *Melaleuca cajuputi* (EEMC) as botanical fungicide and insecticide on *Alternaria* sp. and insect pests (aphids and mealybugs) was conducted in the laboratory. Regarding antifungal activity, the results were revealed that all formulations of EECV+EEMC (2500 ppm concentration of extract mixture in the ratios of 100:0, 75:25, 50:50, 25:75 and 0:100) gave the inhibition effect on mycelial growth of the tested fungus in the ranges of 27.5-92.75%. Whereas the spore germination test showed the effect in the ranges of 75-100%. However, the best inhibitory effects were noted in EEMC alone treatment while EECV gave the lower inhibition effect. For extract mixtures, the inhibition effect increased with the increasing concentrations of EEMC. Our findings agreed with many previous researches. Both *C. viminalis* and *M. cajuputi*, belong to Myrtaceae family, presented strong inhibition effect on plant pathogenic fungi (Bharat and Praveen, 2016; Pawar and Thaker, 2007; Siddique *et al*., 2018; Thanaboripat *et al*., 2007; Thanaboripat *et al*., 2016; Somnuek *et al*., 2020). Apparently, our tested extracts were strongly inhibitory to *Alternaria* spore germination. Abnormal fungal spores were found in EEMC alone and the extract mixed with EEMC. The antifungal effects of EECV and EEMC found in our study may be attributed to the presence of their phytochemical compounds. The chemical compounds such as phenolics, flavonoids, tannins and terpenoids were presented in the Myrtaceae plants. These phytochemicals have the ability to suppress plant pathogenic fungi e.g., to inhibit enzymes, bind adhesins and proteins, and degrade cell walls (Tiwari *et al*., 2011; Gurjar *et al*., 2012).

Many extracts and essential oils from plants including the Myrtaceae plants have been reported that it gave mortality and repellent effects on insect pests. Our obtained result implied that EECV and EEMC exhibited mortality effects on both aphids and mealybug. The highest mortality effect (100%) was noted in EECV and EEMC treatment at 20000 ppm concentrations. However, EEMC showed an effect more than EECV which gave the LC_{50} values on both tested insects with the range of 2671-2981 and 2541-3801 ppm. Our finding agreed with previous researchers who reported that Myrtaceae plants could kill and repel various insect pests (Ndomo *et al*., 2009; Majeed *et al*., 2018; Sannongmueang *et al*., 2018). Besides, Sannongmueang *et al*. (2018) found hexane, acetone and ethanolic crude extracts from *Callistemon lanceolatus* at 1-5% (w/v) showed the toxic and repellent activities against mealybug. While Visheentha *et al*. (2018) reported hexane, dichloromethane and methanol extracts from *M. cajuputi* stem gave mortality effect on *Camponotus* sp. Showler (2017) reported that essential oils from *Syzygium*, *Melaleuca* and *Eucalyptus* (Myrtaceae) presented the effect on horn flies and stable flies. In this regard, the effectiveness of plant extracts and essential oils on mortality and repellency of insect pests would be related to the phytochemical components. Such phenols, flavonoids, alkaloids and terpenoids are important compounds containing in the plant extract. Those bioactive compounds showed broad-spectrum action or multiple modes of action, including antifeedant, repellency and toxicity on insect pests (Showler, 2017; Niroumand *et al*., 2016; Mohamed *et al*., 2017; Hematpoor *et al*., 2017; Hikal *et al*., 2017).

In conclusion, ethanolic crude extracts from *C. viminalis* (EECV) and *M. cajaputi* (EEMC) alone and extract mixtures of both plants (75:25, 50:50 and 25:75) significantly showed the inhibition effect on mycelial growth and spore germination of *Alternaria* sp. with the ranges of 27.5-92.7% and 75-100%, respectively. Also, the effect in terms of toxic and repellent activities of both plants on aphids and mealybug, all tested concentrations of both plant extracts gave mortality and repellency effect on tested insects in ranges of 38-100% and 14-100%, respectively. Therefore, EECV and EEMC could be used as botanical fungicide and insecticide for controlling *Alternaria* sp. and insects (aphid and mealybug) in laboratory conditions. However, these two plant extracts should be further tested in greenhouse and field conditions in order to obtain the botanical pesticide as an eco-friendly product.

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(Received: 16 August 2021, accepted: 30 October 2021)