Hidden synergistic effects of the combinations of plant extracts against plant pathogenic fungi

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ABSTRACT

In this study, we determined the *in vitro* antifungal activities of five plant crude extracts, namely *Acorus calamus* L., *Coscinium fenestratum* (Goetgh.) Colebr., *Piper betle* Linn., *P. nigrum* L. and *P. retrofractum* Vahl. Both individually and in combinations in seven different ratios and in concentrations of 10,000 and 1,000 ppm against the radial growth of *Alternaria brassicicola*, *Colletotrichum capsici* and *Fusarium oxysporum* f.sp. *cubense*. The findings indicated that the synergistic activities of the combinations were observed more in the lower concentration tested (1,000 ppm) than in the higher concentration (10,000 ppm) at different ratios. The synergistic activities of the combinations of two extracts against *A. brassicicola* were increased from 34.29% to 67.14% when tested at 1,000 ppm. Antagonistic effects (14.29%) were found when tested at 10,000 ppm but were not observed when tested at 1,000 ppm. At 10,000 ppm, the combined extracts different ratios displayed synergistic (74.29%) and additive activities (25.71%) against *C. capsici*. At 1,000 ppm, strong synergistic interactions against *C. capsici* were observed in all combinations. In testing effects of the combinations against *F. oxysporum* f.sp. *cubense*, we found that the combined extracts at 10,000 ppm exhibited synergistic (48.57%), additive (31.43%), in different (2.86%) and antagonistic effects (17.14%) but only synergistic and additive activities were detected when tested at 1,000 ppm. Moreover, some crude extracts had no activity (0% MGI) against the fungi tested at 1,000 ppm when tested individually but displayed synergistic effects when combined with another extract, evidenced by the percent mycelial growth inhibition of the combination mixtures. The results in this study indicate that the combination of plant extracts has promising synergistic activity against plant pathogens and that the concentration is a key factor in evaluating the effects. These results provided useful information as a guideline for further evaluation of the effects of these extracts against plant diseases in greenhouse and field studies.

Keywords: synergism; plant extracts; botanical fungicides; antifungal activity

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INTRODUCTION

Fungicide application has been the main approach to control and manage of plant diseases in crop production (Thind, 2012). However, the overuse of fungicides and repetitive fungicide applications has resulted in pathogen resistance and disease outbreaks (Thind, 2012; Ishii and Hollomon, 2015). *Alternaria brassicicola*, *Colletotrichum capsici* and *Fusarium oxysporum* f.sp. *cubense* are considered as the most devastating foliar and fruit disease pathogens and have caused loss of yield both qualitatively and quantitatively in vegetable and fruit production worldwide (Nowicki *et al*., 2012; Heck *et al*., 2017; Mostert *et al*., 2017; Suwannarat *et al*., 2017). These three fungi are soil- and airborne pathogens affecting economically important plants. *Alternaria brassicicola* is the causal

agent of leaf spot, leaf blight and wilt on various hosts in Brassicaceae (Amein *et al*., 2011; Nowicki *et al*., 2012). *Colletotrichum capsici* causes a well-known disease, anthracnose, in chili (Suwannarat *et al*., 2017). *Fusarium oxysporum* f.sp. *cubense* causes vascular wilt, or panama disease, in banana (*Musa* spp.) (Xue *et al*., 2015). Various groups of single-site and multi-site fungicides have been recommended for control of these fungi in fields, including demethylation inhibitors, benzimidazoles, and dicarboximides (Krämar and Schirmer, 2007). However, many scientists have attempted to find alternatives to synthetic fungicides by investigating plant extracts and biological control agents. Medicinal plants have proved to be secondary metabolite producers, some of which possess a broad spectrum of biological activities such as antiviral, antibacterial, antifungal activities (Johnny *et al*., 2011; Alves *et al*., 2015; Dethoup *et al*., 2018).

Normally, the *in vitro* classical protocols for studies of antifungal metabolites from plants against plant pathogens include 1. Extraction of plant metabolites with organic solvents, 2. Evaluation of the antifungal effect of plant extracts on mycelial growth of plant pathogens *in vitro* and 3. Isolation and classification of key antifungal compounds by chromatographic techniques and antifungal assays. However, bioactive metabolites of many of potent plant extracts which possess strong antifungal activities against plant pathogens have not been isolated and classified because these bioactive metabolites are produced in small amounts or they do not produce an effect alone but through the synergistic action of two or more compounds in each plant extract (Mahlo *et al*., 2016).

Moreover, many studies have found that the combination of plant extracts or plant extract combined with antibiosis showed synergistic activity against pathogens. For example, Medina-López *et al*. (2016) reported the synergistic activities of a mixture of two antifungal fractions obtained from *Baccharis glutinosa* and *Jacquinia macrocarpa* extracts against *Aspergillus flavus* and *Fusarium verticillioides*. Da Rapper *et al*. (2012) reported a strong synergistic effect of the combined use of essential oils derived from *Boswellia papyrifera* and *Commiphora myrrha* against *Cryptococcus neoformans* and *Pseudomonas aeruginosa*. In addition, many studies have reported that plant extracts and different antibiotics displayed significant synergistic effects against human pathogens (Chusri *et al*., 2015; Farooqui *et al*., 2015; Wang *et al*., 2016; Zuo *et al*., 2018). However, these studies of synergistic effects of combinations of either plant extracts or antimicrobial agents have focused on human pathogens and pharmaceutical applications whereas tests of such effects on plant pathogenic fungi are still limited.

These previous reports confirmed that there are the synergistic effects between secondary metabolites produced by plants. In a previous study, it was reported the efficacy of 10 crude extracts of Thai medicinal plants against Alternaria Chinese kale black spot. We found that the extracts of *Coscinium fenestratum* and *Piper betle* showed a strong antifungal activity against *A. brassicicola*, causing 80% Chinese Kale black spot disease suppression in green house tests and other crude extracts displayed low to medium activities against this disease (Dethoup *et al*., 2018). We hypothesized that applying combinations of the plant crude extracts may exploit synergistic effects against *A. brassicicola* and other plant pathogenic fungi. The findings of this study have led us to explore the potential of combining plant extracts for applications which are cost-effective and produced from easily found plant. Hence, the aims of this study were to evaluate the *in vitro* effects of the combinations of plant extracts in different ratios and concentrations on the radial growth of three plant pathogenic fungi namely, *A. brassicicola, C. capcisi* and *F. oxysporum* f.sp. *cubense*.

MATERIALS AND METHODS

The five plants used in this study, namely *Acorus calamus, Coscinium fenestratum*, *Piper betle*, *P. nigrum* and *P. retrofractum* were purchased from a medicinal plant market, "Saphanmai," in Bangkok, Thailand (Table 1).

A voucher specimen of each plant was deposited with the Natural Products for Plant Protection Division, Kasetsart University, Thailand.

Extraction

Crude extracts of five medicinal plants were prepared as previously described by Dethoup *et al*. (2018). Briefly, dried fine powder made from each plant was extracted thrice with 70 % ethanol at 28 ± 3°C. The ethanol extract of each plant was filtered through three layers of sterile cheese cloth and then concentrated at reduced pressure to give a crude ethanol extract of each plant.

Antifungal Activity Bioassays

The dilution plate method was used to evaluate the effects of the combinations of two extracts against three plant pathogenic fungi. Briefly, each crude ethanol extract was dissolved in dimethyl sulfoxide (DMSO) and serially diluted in sterile water to prepare stock solutions of 100,000 and 10,000 ppm. Then, combinations of two crude extracts were mixed at seven ratios of 1:1 1:2 1:3 1:4 4:1 3:1 and 2:1; after that, each combination was mixed with warmed potato dextrose agar (PDA) to obtain final concentrations of 10,000 and 1,000 ppm, and poured in a separate sterile Petri dish. Each crude extract was mixed with distilled sterile water at seven ratiosof 1:1 1:2 1:3 1:4 4:1 3:1 and 2:1, mixed with warmed PDA, poured in a separate sterile Petri dish to obtain the final concentrations of 10,000 and 1,000ppm, and used as negative controls. Afterward, a mycelial plug (5 mm. in diam.) each from the periphery of a 7-day-old pathogenic fungus colony was placed on the center of each PDA plate containing combination of two crude extracts and incubated at 25°C for 10 days. The mycelial growth inhibition (MGI) was calculated according to the formula: %MGI = (R1–R2)/R1*100, where R1 = the diameter of plant pathogenic fungus mycelial growth of the negative control, R2 = the diameter of plant pathogenic fungus mycelial growth in Petri dishes treated with extracts. Each assay was performed in five replications and repeated in triplicate. The combination effect index (CEI), which was modified from fractional inhibitory concentration index described by Farooqui *et al*. (2015), was used to analyze interactions in this study,where >3 synergy; 2 to 3 additive effect; 2 indifferent or no effect; and < 2 antagonism.

$$CEI = CEI(A) + CEI(B) = \frac{\%MGI \text{ of A in combination}}{\%MGI \text{ of A tested alone}} + \frac{\%MGI \text{ of B in combination}}{\%MGI \text{ of B tested alone}}$$

Where % MGI of A and B was the % mycelial growth inhibition of each combination.

Statistical analysis

All experiments in this study were conducted at least twice. Since there was no significant difference between the repetitions of each experiment, the separate data of each experiment were pooled and then submitted to Analysis of Variance (ANOVA), and means were compared by Duncan's Multiple Range test ($P < 0.05$), using the statistical program SPSS version 19 (IBM Corporation, Somers, NY).

RERULTS

Antifungal activity of individual extracts

When testing the five crude plants extracts individually *in vitro*, the reduction of the mycelial growth of all plant pathogenic fungi tested was concentration-dependent (Table 1). The results showed that the crude extracts of *C. fenestratum* and *P. betle* showed great antifungal activity against *A. brassicicola* with 100% mycelial growth inhibition at the higher concentration tested and *P. retrofractum* > *P. nigrum* > *A. calamus* at 1,000 ppm.

However, each extract displayed less antifungal activity against the radial growth of *C. capsici* than that of *A. brassicicola* inhibition. The results showed that the crude extract of *P. betle* had the best activity against *C. capsici* with 100% inhibition at both concentrations tested whereas *P. nigrum* extract displayed no activity on radial growth of *C. capsici* at 1,000 ppm. Besides showing great antifungal activity against *A. brassicicola* and *C. capsici*, the extract of *P. betle* also exhibited the best mycelial growth inhibition of *F. oxysporum* f.sp. *cubense* at both concentrations tested

***In vitro* antifungal activity**

The effects of the combined extracts in seven

different ratios at 10,000 ppm against *A. brassicicola* are shown in Tables 2-3. The types of interaction found were characterized as synergistic, additive (41.42%), non-interactive (10%) and antagonistic (14.29%). At 1,000 ppm, the interactions were characterized as synergistic (67.14%), additive (31.43%) and non-interactive (1.43%) effects; and no antagonistic interaction was found (Table 3). At 1,000 ppm, the combinations of *P. betle*:*P. retrofractum*, *P. betle*:*A. calamus*, *C. fenestratum*:*P. nigrum* and *C. fenestratum*: *A. calamus* exhibited synergistic activity at all ratios. Moreover, the combination of *P. nigrum*: *A. calamus* at ratios of 1:1 2:1 3:1 and 4:1 displayed significant ($P< 0.5$) synergistic effects against *A. brassicicola* with the CEI values in the range of 4.42-4.82.

At 10,000 ppm, the combined extracts in seven different ratios displayed synergistic (74.29%) and additive interactions (25.71%) against *C. capsici* (Table 4) with no indifferent or antagonistic interactions. The combinations of *P. betle*: *C. fenestratum* and *C. fenestratum*: *P. retrofractum* displayed synergistic activity against this fungus at all ratios. At 1,000 ppm, strong synergistic interactions against *C. capsici* were observed in all combinations (Table 5). However, some crude extracts showed no activity against the fungi tested at the lower concentration; hence, we could not calculate the CEI values even though the combinations displayed synergistic effects evidenced by the fact that the percent mycelial growth inhibition of the combinations was higher than zero. Strong synergistic activities against *C. capsici* were detected in the combinations of *P. betle*:*P. nigrum*, *C. fenestratum*: *P. nigrum* and *P. betle*:*A. calamus* at 1:1 and 1:2 at 10,000 ppm. Interestingly, the best synergistic interactions were observed in the combination of *P. betle*: *P. retrofractum* at 1:1 and 1:2 at 1,000 ppm, for which the CEI values rose respectively to 13.61-14.05.

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Table 1. Plants used in this study and the efficacy of their crude ethanol extracts against *Alternaria brassicicola* using the dilution plate method

Plant extract	Plant part used	Concentration (ppm)	Plant Pathogenic Fungi		
			Alternariabrassicicola	***Colletotrichumcapsici***	***Fusariumoxysporum*f.sp.*cubense***
Acoruscalamus	Rhizomes	10,000	88.26	87.29	76.25
		1,000	40.52	42.22	26.94
Coscinium fenestratum	Stems	10,000	100	55.28	50.56
		1,000	100	22.43	30.77
Piper betle	Leaves	10,000	100	100	100
		1,000	100	73.75	60.56
Pipernigrum	Fruits	10,000	79.72	49.86	48.61
		1,000	55.91	0	32.78
Piper retrofractum	Rruits	10,000	74.17	59.72	72.22
		1,000	57.78	22.22	33.56

Table 2. The *in vitro* CEI values of combinations of plant extracts against *A. brassicicola* at 10,000 ppm

Mixture	Ratio						
	1:1	**1:2**	**1:3**	**1:4**	**4:1**	**3:1**	**2:1**
P. betle: *C. fenestratum*	2^{n}*	2^{n}	2^{n}	2^{n}	2^{n}	2^{n}	2^{n}
P. betle: *P. retrofractum*	2.63^{i-l}	2.47^{k-m}	2.38^{lm}	2.21^{mn}	**3.25^{c-e}**	**3.05^{d-f}**	2.68^{h-l}
P. betle: *P. nigrum*	**3.35^{cd}**	2.45^{k-m}	2.06^{n}	1.96^{n}	**4.97^{a}**	**4.30^{b}**	**3.48^{c}**
P. betle: *A. Calamus*	2.82^{f-j}	2.82^{f-j}	2.70^{g-j}	2.64^{h-l}	**4.10^{b}**	**3.25^{c-e}**	2.99^{e-g}
C. fenestratum: *P. retrofractum*	2.88^{f-l}	2.64^{h-l}	2.55^{j-l}	2.41^{j-m}	**3.47^{c}**	**3.35^{cd}**	2.94^{f-h}
C. fenestratum: *P. nigrum*	**3.35^{cd}**	2.52^{j-l}	2.37^{lm}	2.27^{l-n}	**4.97^{a}**	**4.30^{b}**	**3.48^{c}**
C. fenestratum: *A. calamus*	2.82^{f-j}	2.74^{g-k}	2.70^{g-j}	2.64^{h-l}	**4.10^{b}**	**3.25^{c-e}**	2.99^{e-g}
P. retrofractum: *P. nigrum*	1.73^{qr}	1.61^{r}	1.50^{rs}	1.25^{s}	**3.08^{g-l}**	2.75^{j-l}	2.05^{op}
P. retrofractum: *A. calamus*	**3.69^{de}**	2.33^{no}	1.95^{pq}	1.81^{p-r}	**5.57^{a}**	**4.60^{b}**	**3.92^{cd}**
P. nigrum: *A. calamus*	2.92^{h-k}	1.86^{p-r}	1.76^{qr}	1.56^{rs}	**5.68^{a}**	**4.09^{c}**	**3.30^{f-h}**

Synergistic activity is indicated with figures in bold. *Means ± standard errors followed by the same letter were not significantly different at $P<0.05$, when analyzed using Duncan's multiple range testof One-Way ANOVA

The interactions of the combined extracts in seven different ratios against *F. oxysporum* f.sp. *cubense* at 10,000 ppm are shown in Table 6. The interactions found were synergistic (48.57%), additive (31.43%), in different (2.86%) and antagonistic (17.14%). At 1,000 ppm, strong synergistic interactions against this fungus were observed in all combinations except *P. betle*: *C. fenestratum* (1:1) and *P. retrofractum*: *P. nigrum* (4:1, 3:1), which exhibited additive interaction. Interestingly, the combination of *P. retrofractum*: *P. nigrum*, which showed antagonistic interaction in all ratios at 10,000 ppm, displayed synergistic interactions at some ratios when the concentration was 1,000 ppm.

Table 3. The *in vitro* CEI values of combinations of plant extracts against *A. brassicicola*at 1,000 ppm

Mixture	ratio						
	1:1	**1:2**	**1:3**	**1:4**	**4:1**	**3:1**	**2:1**
P. betle: C. fenestratum	2^{q*}	2.12^{pq}	2.19^{o-q}	**3.79^{e-h}**	2.54^{n}	2.47^{no}	2.42^{n-p}
P. betle: P. retrofractum	**4.07^{c-e}**	**3.93^{d-f}**	**3.50^{h-j}**	**3.32^{j-l}**	**4.90^{a}**	**4.44^{b}**	**4.22^{b-d}**
P. betle: P. nigrum	2.96^{m}	**4.94^{a}**	**4.04^{c-f}**	**3.41^{jk}**	**3.58^{g-j}**	2.46^{no}	2.28^{h-q}
P. betle: A. calamus	**4.07^{c-e}**	**3.74^{f-i}**	**3.94^{d-f}**	**3.77^{e-i}**	**4.50^{b}**	**3.40^{jk}**	**3.45^{i-k}**
C. fenestratum: P. retrofractum	**3.30^{j-l}**	**3.16^{k-m}**	2.97^{m}	**3.14^{k-m}**	**4.32^{bc}**	**3.83^{e-g}**	**3.26^{j-m}**
C. fenestratum: P. nigrum	**3.99^{d-f}**	**3.90^{d-f}**	**3.66^{f-i}**	**3.02^{lm}**	**5.27^{a}**	**4.50^{b}**	**4.21^{b-d}**
C. fenestratum: A. calamus	**3.45^{i-k}**	**3.57^{g-j}**	**3.31^{j-l}**	**3.03^{lm}**	**3.05^{lm}**	**4.04^{c-f}**	**4.02^{c-f}**
P. retrofractum: P. nigrum	2.60^{lm}	2.58^{lm}	2.08^{n}	2.05^{n}	**4.41^{a}**	**3.55^{de}**	**3.03^{g-j}**
P. retrofractum: A. calamus	2.69^{k-m}	2.86^{i-l}	2.68^{k-m}	2.53^{m}	2.47^{m}	**3.09^{g-i}**	2.72^{j-m}
P. nigrum: A. calamus	**4.81^{a}**	**3.60^{de}**	**3.26^{e-h}**	2.88^{i-l}	**4.76^{a}**	**4.42^{a}**	**4.58^{a}**

Synergistic activity is indicated with figures in bold. *Means ± standard errors followed by the same letter were not significantly different at $P<0.05$, when analyzed using Duncan's multiple range test of One-WayANOVA

Table 5. The *in vitro* CEI values of combinations of plant extracts against *C. capcisi* at 1,000 ppm

Mixture	ratio						
	1:1	**1:2**	**1:3**	**1:4**	**4:1**	**3:1**	**2:1**
P. betle: C. fenestratum	**-***				**4.13^{k}***	**4.31^{k}**	**5.84^{g}**
P. betle: P. retrofractum	**13.61^{b}**				**7.85^{e}**	**9.49^{c}**	**14.05^{a}**
P. betle: P. nigrum							
P. betle: A. calamus	**9.35^{c}**				**4.85^{j}**	**4.76^{j}**	**8.98^{d}**
C. fenestratum: P. retrofractum					**5.47^{h}**	**6.05^{g}**	**6.74^{f}**
C. fenestratum: P. nigrum							
C. fenestratum: A. calamus					**3.16^{m}**	**3.78^{l}**	**5.15^{i}**
P. retrofractum: P. nigrum							
P. retrofractum: A. calamus					**7.70^{e}**	**9.01^{cd}**	**9.07^{cd}**
P. nigrum: A. calamus							

-* could not be calculated due to the percent radial growth inhibition of plant extract(s) alone in the combinations being zero.

Synergistic activity is indicated with figures in bold.

*Means ± standard errors followed by the same letter were not significantly different at $P<0.05$, when analyzed using Duncan's multiple range testof One-Way ANOVA

Table 6 The *in vitro* CEI values of combinations of plant extracts against *F. oxysporum* f.sp. *cubense* at 10,000 ppm

Mixture	ratio						
	1:1	1:2	1:3	1:4	4:1	3:1	2:1
P. betle: *C. fenestratum*	**3.57^{j_l}***	**3.68^{h_k}**	**4.27^{c_f}**	**4.48^{bc}**	**3.02^{n_r}**	**3.12^{m_p}**	**3.31^{l_n}**
P. betle: *P. retrofractum*	2.85^{p_v}	2.91^{p_u}	**3.94^{f_h}**	**4.32^{c_e}**	2.41^{w}	2.71^{r_w}	2.78^{q_w}
P. betle: *P. nigrum*	**3.47^{j_l}**	**3.83^{g_i}**	**4.68^{b}**	**5.27^{a}**	2.02^{x}	2.43^{w}	2.65^{t_w}
P. betle: *A. calamus*	2.73^{r_w}	2.91^{p_u}	**3.76^{h_j}**	**3.54^{i_l}**	2.57^{vw}	2.61^{s_w}	2.67$_{s_w}$
C. fenestratum: *P. retrofractum*	**3.33^{l_n}**	**3.68^{h_k}**	**4.13^{d_f}**	**4.43^{b_d}**	2.73^{r_w}	**3.00^{n_s}**	**3.29^{l_o}**
C. fenestratum: *P. nigrum*	**3.29^{l_o}**	2.83^{p_v}	2.41^{w}	**3.28^{l_o}**	1.48^{x}	1.57^{x}	2.63^{t_w}
C. fenestratum: *A. calamus*	**3.82^{g_i}**	**3.99^{e_h}**	**4.48^{bc}**	**4.59^{bc}**	2.96^{o_t}	**3.10^{m_q}**	**3.42^{k_m}**
P. retrofractum: *P. nigrum*	1.08^{op}	1.15^{no}	1.55^{m}	1.16^{no}	0.79^{p}	1.04^{op}	1.16^{no}
P. retrofractum: *A. calamus*	2.56^{k}	**2.79^{i_k}**	**3.50^{ef}**	**3.89^{cd}**	1.44^{mn}	2.00^{l}	2.15^{l}
P. nigrum: *A. calamus*	2.55^{k}	**3.11^{g_i}**	**3.68^{de}**	**3.89^{cd}**	1.31^{mn}	1.60^{lm}	2.05^{l}

Synergistic activity is indicated with figures in bold. *Means ± standard errors followed by the same letter were not significantly different at $P<0.05$, when analyzed using Duncan's multiple range test of One-Way ANOVA

Table 7. The *in vitro* CEI values of combinations of plant extracts against *F. oxysporum* f.sp. *cubense* at 1,000 ppm

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Mixture	ratio						
	1:1	1:2	1:3	1:4	4:1	3:1	2:1
P. betle: *C. fenestratum*	2.97^{j}*	**-***				**6.77^{d}**	
P. betle: *P. retrofractum*					**5.70^{f}**	**7.88^{b}**	
P. betle: *P. nigrum*					**4.15^{i}**	**5.90^{e}**	
P. betle: *A. calamus*					**4.51^{h}**	**6.83^{d}**	
C. fenestratum: *P. retrofractum*					**4.57^{h}**	**5.29^{g}**	**7.12^{c}**
C. fenestratum: *P. nigrum*					**5.35^{g}**	**5.63^{f}**	**8.13^{ab}**
C. fenestratum: *A. calamus*					**4.00^{i}**	**5.38^{g}**	**9.22^{a}**
P. retrofractum: *P. nigrum*	**3.59^{gh}**	**6.52^{c}**			2.70^{i}	2.93^{i}	**3.79^{fg}**
P. retrofractum: *A. calamus*	**3.51^{h}**	**6.61^{c}**			**3.52^{h}**	**3.79^{fg}**	**5.41^{d}**
P. nigrum: *A. calamus*	**5.66^{cd}**	**5.00^{d}**			**4.77^{de}**	**5.96^{cd}**	**7.82^{ab}**

-* could not be calculated due to the percent radial growth inhibition of plant extract(s) alone in the combinations being zero.

Synergistic activity is indicated with figures in bold.

*Means ± standard errors followed by the same letter were not significantly different at $P<0.05$, when analyzed using Duncan's Multiple Range Test of One-Way ANOVA

DISCUSSION

The five medicinal plant extracts were tested individually and in combination at the concentrations of 10,000 ppm and 1,000 ppm to determine the effects on the radial growth of three plant pathogenic fungi, namely, *A. brassicicola*, *C. capsici*, and *F. oxysporium* f.sp. *cubense* using the dilution plate method. The results showed that the extracts possessed antifungal activity in a concentration-dependent manner and that this varied with the fungal species tested. *A. brassicicola* was the most sensitive followed by *C. capsici* and *F. oxysporium* f.sp.*cubense* respectively, when each extract was tested individually. *Piper betle* extract exhibited the best antifungal activity against all plant pathogenic fungi tested, which corresponded to previous reports (Johnny *et al.*, 2011; Singburaudom, 2015). However, the effects of combinations of the extracts against plant pathogenic fungi have not been reported. To the best of our knowledge, this is the first report of the *in vitro* effects against plant pathogenic fungi of

combinations of *A. calamus*, *C. fenestratum*, *P. betle*, *P. nigrum* and *P. retrofractum* crude extracts.

Previous studies reported the synergistic activities of combinations of plant extracts and antibiotics, plant oils and antibiotics, antibiotics and antibiotics against human pathogens, evidenced by the lower MIC values in the combinations treatment than those of their MIC values in individually tests (Betts *et al*., 2013; Chusri *et al*., 2014; Wang *et al*., 2016; Carvalho *et al*., 2018). Most studies have used the MIC value of each extract to assess their synergistic interactions in combinations and to assessthe FIC index as proposed by several authors (Didry *et al*., 1993; Van Vuuren and Viljoen, 2011; Farooqui *et al*., 2015; Medina-López *et al*., 2016). In this study, we tested the interactions between the combined extracts at two concentrations: 10,000 ppm and 1,000 ppm, to assess the *in vitro* optimal ratio and concentration; we found that the synergistic activity was higher in the lower concentration than that of the higher concentration. Results from Tables 2 and 3 showed that the synergistic activity against *A. brassicicola* was increased from 34.29% to 67.14% when tested at 1,000 ppm and that antagonistic effects (14.29%) were found when tested at 10,000 ppm, but no antagonistic effect was observed when tested at 1,000 ppm. In testing combinations against *F. oxysporum* f.sp. *cubense*, the combined extracts at 10,000 ppm exhibited synergistic (48.57%), additive (31.43%), in different (2.86%) and antagonism effects (17.14%), but only synergistic and additive activities were detected when tested at 1,000 ppm (Tables 6 and 7).

Moreover, some plant extracts had no antifungal activity against plant pathogens when tested alone at the concentration of 1,000 ppm but displayed inhibitory effects when tested in combinations with other plant extracts at different ratios as shown in Tables 4, 5 and 7 (Data are not displayed because CEI values could not be calculated). We hypothesize that the compounds in each plant extract might interact more readily at low as opposed to high concentrations and also that as plants are well known producers of a varied classes of secondary metabolites, the non-active compounds may interfere with synergetic interactions in a high concentration mixture. Similar to our results, Sun *et al*., (2018) recently reported the potent synergistic effect of the combination of 200 mg/L ε-poly-L-lysine and 400 mg/L chitooligosaccharide against *Botrytis cinerea* in tomatoes. Plant extracts used in this study have been reported to interact synergistically with other plant extract against human pathogens especially *Piper* species extracts. For example, Taukoorah *et al*. (2016) reported the synergistic activity of *P. betle* ethyl acetate extract and chloramphenicol against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *S. Pyogenes* and *Propionibacterium acnes*. Sanjukta *et al*. (2015) studied the effects of combinations of *P. nigrum* and *Allium sativum* extracts against food pathogens and found that their combined extracts displayed strong synergistic effects against tested fungi. Recently, the synergistic antibacterial effects of the combination of *P. peltatum* and *P. marginatum* with nisin against *Alicyclobacillus acidoyerrestris* growth in commercial orange juice was reported by de Pascoli *et al*. (2018). In addition, Aqui*et al*., (2006) reported the synergistic effect of *A. calamus* extract when combined with *Hemides musindicus* and *Plumbago zeylanica* against anti-methicillin-resistant *Staphylococcus aureus*. More recently, Kumar *et al*. (2015) found that asarones from *A. calamus* in combination with azoles and amphotericin B showed potent action against *Candida* spp. i*n vitro*.

Although certain plant extracts have strong antifungal activity against plant pathogenic fungi, some of them might be high in cost orbe available in only limited amounts in markets as a source of botanical fungicide. Hence, the combining of plant extracts might be a promising strategy to find alternative synthetic fungicides against plant diseases as well as to overcome the resistance mechanisms of plant pathogens. In this study, we investigated the *in vitro* antifungal activities of five plant extracts

individually and in combinations at various ratios and at the concentrations of 10,000 ppm and 1,000 ppm against three plant pathogenic fungi. The effects of combinations of these five medicinal plants on plant pathogens have not been previously reported. Our results indicated that more synergistic activity of the combinations at different ratios was observed in the low concentration tested than in the high concentration. The results also showed the hidden potential synergistic activities of some combinations of plant extracts tested in this study against each plant pathogenic fungus. This may be useful information as a guideline for evaluating the effects of these extracts against plant diseases in greenhouse and field studies in terms of cost-effectiveness. However, if these combinations are to be used more widely in agriculture as novel botanical fungicides, evaluation of their effects under greenhouse and field conditions would be needed.

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