Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 309046, 6 pages
doi:10.1155/2012/309046

Research Article

Effect of Crude Leaf Extracts on *Colletotrichum gloeosporioides* (Penz.) Sacc.

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Received 15 April 2012; Accepted 29 May 2012

Academic Editor: Kabkaew Sukontason

Colletotrichum gloeosporioides (Penz.) Sacc. is a fungus that causes anthracnose disease in tropical fruit plants, resulting in damages of the fruit plants and low yield and quality of fruits. The use of chemical fungicides is common for management of this disease, but it also results in the development of fungal resistance to the chemicals. Therefore, this study aims to *in vitro* evaluate the efficacy of 14 crude leaf extracts against *C. gloeosporioides*. The results showed that *Piper sarmentosum* leaf extracts, using 80% of ethanol, methanol, and chloroform as solvents, were found to have very high antifungal activities. Crude methanol extract of *P. sarmentosum* leaves could effectively inhibit the growth of fungal mycelium (100%), followed by crude chloroform extract (81.85%) and 80% ethanol extract (45.50%). Maximum inhibition of *C. gloeosporioides* spore germination could be obtained after application with crude methanol extract of *P. sarmentosum* leaves and crude chloroform extract of *Mentha cordifolia* leaves at 1.25 and 2.5%, respectively. In conclusion, crude extracts of *P. sarmentosum* leaves were found to be highly effective for inhibiting both *C. gloeosporioides* mycelium growth and spore germination, and they have a potential as the new natural fungicides for management of anthracnose disease.

1. Introduction

Colletotrichum gloeosporioides (Penz.) Sacc. is a causative agent for anthracnose disease in many tropical fruit trees such as mango and papaya. This disease is very harmful and can cause spoilage and rotting of fruit plants, resulting in low yield and poor quality of the fruits [1]. The use of chemical fungicides is the most common choice for management of anthracnose disease, but this also causes the development of fungal resistance [2]. In addition, continuous and inappropriate use of chemical fungicides to manage anthracnose disease is not considered to be the long-term solution because this can increase the investment expenses, the risk of having high levels of toxic residues, and also the concerns in human health and environmental settings [3]. Due to these reasons, there are several attempts to search for alternative measures to control the anthracnose disease effectively. Recent efforts have focused on the development of environmentally safe, long-lasting, and effective biocontrol methods for management of anthracnose diseases. The utilization of natural products, especially the plant extracts, has been shown to be effective against many plant pathogens and considered to be safe for consumers and environments [4]. A number of plant species have been reported to possess natural substances that are toxic to a variety of plant pathogenic fungi [5, 6]. The extracts derived from *Curcuma longa* (leaf and rhizome), *Tagetes erecta* (leaf), and *Zingiber officinales* (rhizome) were shown to have antifungal activities against fungal anthracnose by completely inhibiting conidial germination of *C. gloeosporioides* [7]. The aqueous leaf extracts of custard apple (*Annona reticulate* L.) and papaya could inhibit spore formation and germination of *Rhizopus stolonifer* and also conidial formation of *C. gloeosporioide*s [8]. In addition, *C. capsici* mycelial growth

and spore germination were found to be suppressed by crude leaf extracts of *Piper betle* L. using methanol, chloroform, and acetone as solvents [9]. Hence, in this study, the *in vitro* antifungal activities of 14 leaf extracts were evaluated against *C. gloeosporioides*, a causative agent of mango anthracnose disease.

2. Materials and Methods

2.1. Fungal Culture. *C. gloeosporioides* was isolated from the upper surface of infected mango and cultured using potato dextrose agar (PDA) medium at 25°C.

2.2. Plant Materials and Extractions. Leaves from 14 different plant species were collected locally or bought at local markets of Maha Sarakham province which is in the northeast region of Thailand (Table 1). Leaf samples were thoroughly washed using tap water, air-dried at room temperature for 3 to 4 h, and finally dried in a hot-air oven at 45–50°C for 1 to 2 days depending on the plant species. Dried leaf samples were ground using small grinder, then placed in polyethylene bags, and stored at 4°C until required. For each sample, 50 g of leaf powder were added to 150 mL of methanol (M), 80% ethanol (E), or chloroform (F) (thus ratio between leaf powder and solvent was 1 : 3). The mixtures were agitated for 72 h on rotary shaker (130 rpm). The obtained extracts were centrifuged at 8,000 rpm for 10 minutes, filtered through Whatman filter paper no. 1, and transferred to 250 mL round-bottom flasks. Finally, these 42 extracts were evaporated using rotary evaporator at 45°C. Concentrated extracts were allowed to dry in hot-air oven, weighed again, and kept at 4°C until required for antifungal assays.

2.3. Screening of Leaf Extracts against C. Gloeosporioides Mycelial Growth. Forty two crude leaf extracts were *in vitro* tested for their efficacy against *C. gloeosporioides* mycelia growth using the poisoned food technique [10]. All crude leaf extracts were reconstituted to have the concentration of 5%. Then 1 mL of each extract was used for mixing with 19 mL of warm PDA and poured into 9 cm sterile Petri dish. After solidification, the plates were inoculated with the 6 mm agar piece containing a week old *C. gloeosporioides* mycelia. For each crude leaf extracts, the experiments were performed in three replicates. PDA plates mixed with carbendazim (commercial fungicide at 0.005%) and sterile distill water were served as positive and negative controls, respectively. The inoculated plates were incubated at 30°C, and the diameters of fungal colonies were measured every day for 5 days.

Inhibition of mycelial growth was calculated using the following formula [11]:

$$\%\ \text{Inhibition} = \frac{X - Y}{X} \times 100, \quad (1)$$

X: diameter of fungal colony grown on negative control plate, Y: diameter of fungal colony grown on plates containing crude leaf extracts.

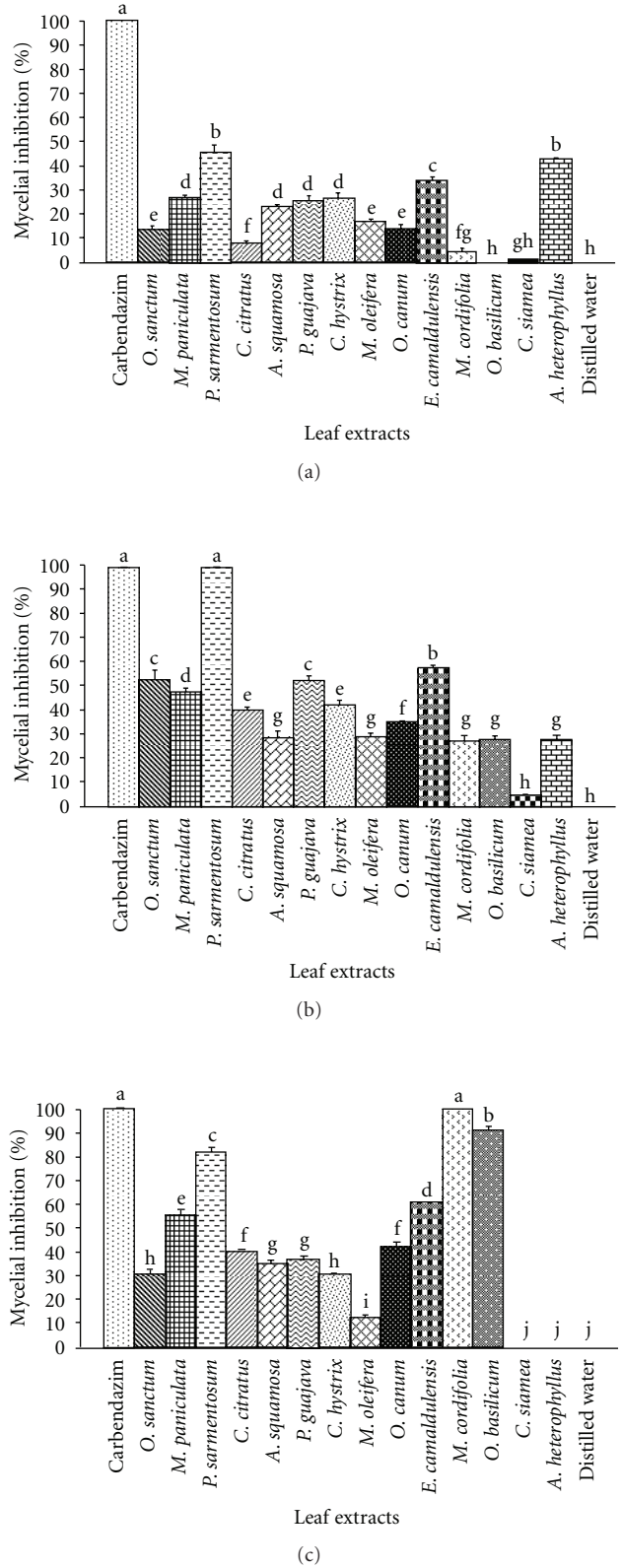

Figure 1: Inhibition of *C. gloeosporioides* mycelial growth by crude leaf extracts using (a) 80% ethanol, (b) methanol, and (c) chloroform as solvents. Bars (mean ± SE) with the same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

Table 1: List of plants.

Scientific name	Family	Common name
Cymbopogon citratus Stapf.	Gramineae	Takhrai, lemongrass
Citrus hystrix DC.	Rutaceae	Leech lime
Murraya paniculata (L.) Jack.	Rutaceae	Orange jessamine, satin-wood
Ocimum basilicum Linn.	Labiatae	Horapa, sweet basil, common basil
Ocimum canum Linn.	Labiatae	Hairy basil
Moringa oleifera Lamk.	Moringaceae	Horse radish tree
Annona squamosa Linn.	Annonaceae	Sugar apple
Ocimum sanctum Linn.	Malvaceae	Holy basil, sacred basil
Psidium guajava Linn.	Myrtaceae	Guava
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Red river gum, red gum
Artocarpus heterophyllus Lam.	Moraceae	Jackfruit tree
Piper sarmentosum Roxb. Ex Hunter.	Piperaceae	Chaplu
Mentha cordifolia Opiz.	Lamiaceae	Kitchen mint, marsh mint
Cassia siamea (Lamk.) Irwin and Barneby	Fabaceae	Cassod tree, siamese senna

2.4. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve of the leaf extracts that were found to have high levels of activities against *C. gloeosporioides* mycelial growth were selected for further testing at lower concentrations. Various concentrations of selected crude leaf extracts were prepared (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and *in vitro* tested against *C. gloeosporioides* mycelial growth (as described above) and spore germination. Inhibition of spore germination was examined by spreading 100 μL of *C. gloeosporioides* spore suspension (10^5 spores/mL) on PDA plates containing each leaf extracts. Carbendazim and sterile distill water were served as positive and negative controls, respectively. Plates were incubated at 30°C and monitored for 7 days.

2.5. Statistical Analysis. All data were subjected to analysis of variance (ANOVA) using the general linear models procedure (SAS Institute, Cary, NC, USA). The data of the percentages of mycelial inhibition were arcsine transformed before analysis. The means of % mycelial inhibition of all treatments were compared and determined using the LSD test at $P \leq 0.05$.

3. Results

3.1. Screening of 42 Crude Leaf Extracts against C. Gloeosporioides Mycelial Growth. Different solvents used for extraction could result in different levels of *in vitro* antifungal activities of the crude leaf extracts (5%) as measured by poisoned food technique. The antifungal activities of leaf extracts using 80% ethanol, methanol, and chloroform as solvents were found to range between 0.77–45.50%, 4.35–100% and 12.37–100%, respectively (Figure 1). Even though all crude leaf extracts exhibited certain levels of activities against *C. gloeosporioides* mycelia, the 80% ethanol extract of *O. bacilicum* and chloroform extracts of *A. heterophyllus* and *C. siamea* did not effectively prevent mycelial growth. *C. siamea* leaves that were extracted using 80% ethanol and methanol were found to have very low antifungal activities at 0.77 and 4.35%, respectively (Figure 1).

Crude leaf extracts using 80% ethanol as solvent were shown to have rather low antifungal activities (less than 50%), as shown by that *P. sarmentosum, A. heterophyllus,* and *E. camaldulensis* could prevent the growth of *C. gloeosporiodes* mycelia at 45.50, 42.75, and 33.85%, respectively (Figure 1(a)). Interestingly, crude methanol extracts of *P. sarmentosum* leaves exhibited the highest inhibition activities against *C. gloeosporioides* mycelialgrowth (100%), followed by *E. camaldulensis* (57.75%), *O. sanctum* (52.75%), and *P. guajava* (52.75%). However, the other methanol leaf extracts were found to have levels of antifungal activities less than 50% (Figure 1(b)). There were 5 chloroform extracts that were found to have more than 50% inhibition activities against *C. gloeosporioides* mycelial growth, including *M. cordifolia* (100%), *P. sarmentosum* (81.75%), *E. camaldulensis* (60.25%), *M. paniculata* (55.50%), and *O. bacilicum* (91.10%) (Figure 1(c)).

3.2. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve crude leaf extracts (derived from 7 plant species), including the extracts of *P. sarmentosum* and *E. camaldulensis* in all solvents, the extract of *A. heterophyllus* in 80% ethanol, the extracts of *O. sanctum* and *P. guajava* in methanol, and the extracts of *O. bacillicum* and *M. paniculata* in chloroform were prepared at various concentrations (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and determined for their efficacy against *C. gloeosporioides* mycelia growth and spore germination.

Although at lower concentrations these plant extracts exhibited lower antifungal activities, some plant extracts remained effective (Tables 2 and 3). In particular, when compared to carbendazim (commercial fungicide), the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* at 2.5% could significantly inhibit the

Table 2: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* mycelial growth.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* mycelial growth* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	54.45 ± 0.71^{cA}	52.75 ± 0.35^{efB}	33.40 ± 0.85^{fC}	17.25 ± 1.06^{fD}	16.50 ± 2.12^{cD}	0.57 ± 0.18^{cdE}	0.00 ± 0.00^{dE}
A. heterophyllus/E	7.45 ± 1.10^{hA}	3.85 ± 0.50^{jkB}	1.95 ± 0.78^{jkC}	0.27 ± 0.03^{hD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}	0.00 ± 0.00^{dD}
O. sanctum/M	3.85 ± 0.21^{iA}	1.12 ± 0.53^{klB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. guajava/M	35.25 ± 1.06^{eA}	20.50 ± 0.70^{iB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
O. basilicum/C	57.25 ± 1.06^{cA}	56.50 ± 0.70^{eA}	36.35 ± 2.33^{eB}	21.50 ± 0.7^{eC}	9.87 ± 0.18^{dD}	0.37 ± 0.17^{deE}	0.00 ± 0.00^{dE}
M. cordifolia/C	97.60 ± 0.84^{aA}	75.75 ± 1.76^{cB}	56.50 ± 2.12^{cC}	37.50 ± 0.7^{cD}	19.12 ± 1.24^{bE}	3.85 ± 0.50^{bF}	37.50 ± 0.71^{cG}
P. sarmentosum/E	16.50 ± 2.12^{gA}	5.30 ± 0.98^{jB}	3.35 ± 0.92^{jB}	0.27 ± 0.03^{C}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. sarmentosum/M	100.00 ± 0.00^{aA}	88.25 ± 1.76^{bB}	80.00 ± 1.41^{bC}	56.50 ± 0.71^{bD}	19.10 ± 2.70^{bE}	3.85 ± 0.51^{bF}	1.37 ± 0.10^{bF}
P. sarmentosum/C	77.75 ± 1.80^{bA}	68.00 ± 2.83^{dB}	42.25 ± 1.06^{dC}	30.85 ± 1.63^{dD}	9.50 ± 2.12^{dE}	0.00 ± 0.00^{eF}	0.00 ± 0.00^{dF}
E. camaldulensis/E	29.75 ± 2.48^{fA}	26.50 ± 4.95^{hB}	23.85 ± 0.21^{gC}	16.75 ± 0.35^{fD}	5.85 ± 0.51^{eE}	0.82 ± 0.71^{cF}	0.00 ± 0.00^{dF}
E. camaldulensis/M	49.50 ± 2.12^{dA}	37.25 ± 1.06^{gB}	11.50 ± 2.12^{iC}	8.85 ± 0.50^{gCD}	7.75 ± 0.35^{deD}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
E. camaldulensis/C	55.25 ± 2.50^{cA}	50.75 ± 1.06^{fB}	15.25 ± 2.47^{hC}	8.35 ± 0.50^{gD}	1.37 ± 0.11^{fE}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{jA}	0.00 ± 0.00^{lA}	0.00 ± 0.00^{kA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

Table 3: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* spore germination.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* spore germination* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	51.10 ± 2.68^{cdA}	42.00 ± 4.24^{deB}	32.35 ± 5.16^{efC}	16.25 ± 3.88^{eD}	13.00 ± 2.82^{eD}	5.00 ± 1.41^{efE}	0.00 ± 0.00^{eE}
A. heterophyllus/E	42.50 ± 2.12^{eA}	38.50 ± 3.50^{efA}	21.50 ± 0.71^{gB}	13.20 ± 2.54^{eC}	4.50 ± 0.71^{fgD}	0.00 ± 0.00^{fE}	0.00 ± 0.00^{eE}
O. sanctum/M	33.35 ± 1.06^{fA}	24.00 ± 1.41^{gB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	10.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
P. guajava/M	50.66 ± 1.83^{cdA}	35.25 ± 5.39^{fB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
O. basilicum/C	65.00 ± 7.07^{bA}	58.60 ± 1.97^{cAB}	47.20 ± 5.37^{dBC}	36.33 ± 0.95^{dC}	22.00 ± 1.41^{dC}	9.00 ± 0.00^{eD}	0.00 ± 0.00^{eD}
M. cordifolia/C	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	85.30 ± 6.64^{bB}	67.00 ± 8.48^{bC}	59.33 ± 3.74^{bC}	42.50 ± 0.71^{cD}	31.12 ± 2.65^{cE}
P. sarmentosum/E	56.75 ± 3.88^{cA}	48.00 ± 2.83^{dB}	37.30 ± 1.83^{eC}	20.00 ± 2.83^{eD}	12.20 ± 1.13^{eE}	8.00 ± 1.42^{eE}	0.00 ± 0.00^{eF}
P. sarmentosum/M	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	90.50 ± 6.36^{bB}	72.20 ± 5.94^{bC}	65.15 ± 6.85^{bD}	57.00 ± 8.48^{bD}	36.10 ± 5.51^{bE}
P. sarmentosum/C	100.00 ± 0.00^{aA}	87.50 ± 2.12^{bB}	66.70 ± 3.25^{cC}	55.60 ± 1.97^{cD}	28.00 ± 5.65^{cE}	19.12 ± 1.24^{dF}	14.00 ± 5.66^{dF}
E. camaldulensis/E	48.10 ± 5.15^{deA}	41.60 ± 4.80^{efA}	31.20 ± 1.13^{efB}	18.00 ± 1.41^{eD}	10.50 ± 0.71^{eD}	4.00 ± 1.42^{efDE}	0.00 ± 0.00^{eE}
E. camaldulensis/M	54.35 ± 5.16^{cdA}	43.33 ± 2.36^{deB}	29.50 ± 0.71^{fC}	18.20 ± 1.13^{eD}	10.00 ± 1.41^{efE}	0.00 ± 0.00^{fF}	0.00 ± 0.00^{eF}
E. camaldulensis/C	47.30 ± 3.81^{deA}	40.00 ± 4.24^{efA}	28.16 ± 2.60^{fgB}	17.00 ± 1.41^{eBC}	8.00 ± 0.00^{efCD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{gA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{gA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

growth of *C. gloeosporioides* mycelium at 100 and 97.60%, respectively (Table 2); moreover, both of these plant extracts at 1.25% also completely prevented *C. gloeosporioides* spore germination (100%) (Table 3). However, the other crude leaf extracts at lower concentrations did not exhibit significant antifungal activity against *C. gloeosporioides* (Tables 2 and 3).

4. Discussion

In this study, 12 leaf extracts (obtained from 7 plant species) that were found to inhibit *C. gloeosporioides* mycelial growth very strongly at 5% were selected, then prepared at lower concentrations, and used for further evaluation against

C. gloeosporioides mycelial growth and spore germination (Tables 2 and 3). At 2.5%, the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* were shown to inhibit *C. gloeosporioides* mycelial growth, and at 1.25%, both of these could also completely prevent *C. gloeosporioides* spore germination.

From previous reports, there are a variety of plant extracts that were used to control fungal anthracnose. For instance, crude methanol, chloroform, and acetone extracts of *Piper betle* leaves at the concentration of 10 μg/mL could inhibit the growth of *Colletotrichum capsici* (responsible for anthracnose disease in pepper) mycelium at 85.25, 78.53, and 73.58%, respectively [9]. Also, at the same concentration, crude methanol, chloroform, and acetone extracts of these *P. betle* leaves were found to prevent *C. capsici* spore germination at 80.93, 74.09, and 72.91%, respectively [9]. Moreover, the leaf extracts of *O. bacillicum* and *Allium sativum* exhibited 100% inhibition of *C. gloeosporioides* (responsible for anthracnose in para rubber) mycelial growth when applying at 50 and 100% w/v, respectively, and both of these extracts could completely suppress spore germination when applying as minimal as 10% w/v [12]. Furthermore, the ethanol extracts of *Ocimum gratissimum* and *Aframomum melegueta* leaves were shown to inhibit the growth of *Botryodiplodia theobromae* mycelium (causative agent of banana anthracnose) at 72.1 and 68.2%, respectively [13].

Other plant pathogenic fungi could also be inhibited by plant extracts. For example, the ethanol extracts of *O. gratissimum* and *A. melegueta* leaves were also reported to prevent *Fusarium oxysporum* and *Aspergillus niger* spore germination at over 65% [13]. In addition, *Rhizopus oryzae* spore germination and mycelia growth were found to be suppressed by the leaf extracts of *O. gratissimum* [14].

This study showed that *P. sarmentosum* and *M. cordifolia* leaves had significant antifungal activity. The studies of phytochemical characteristics showed that bioactive compounds in *Mentha* sp. are sitosterol and β-sitosteryl-β-D-glucoside, and in *Piper* sp. are lignans, steroids, neolignans, alkaloids, propenylphenols, terpenes, piperolides, chalcones, flavanones, flavones, and amides bearing isobutyl, pyrrolidine, dihydropyridine, and piperidine moieties, all of which could exhibit high antimicrobial and antifungal properties [15–17]. The levels of plant bioactive compounds with antifungal activity could be influenced by many factors which include the age of plant, harvesting time point, extraction solvent, and method of extraction [18].

In conclusion, this study shows that crude leaf extracts of *P. sarmentosum* have strong antifungal activities against *C. gloeosporioides*. This may suggest their potential for future formulation into products for controlling anthracnose diseases of mango and other fruits. More extensive study of their phytochemical characteristics and *in vivo* efficacy remains to be determined.

Acknowledgments

This work was financially supported by the NRCT, Thailand. The authors also thank the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand, for laboratory equipments and facility.

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