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Detecting the antifungal activity of the piper genus against *Colletotrichum capsici*, the cause of chili anthracnose

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ABSTRACT

This study investigated the antifungal activities of plant extracts that inhibit *Colletotrichum capsici*. Four species of the Piper genus were assessed for their potential as effective natural antifungal agents against chili pathogens. Plant extracts were tested *in vitro* at concentrations of 5, 10, and 20 mg/mL using the poisonous food diffusion technique; they effectively inhibited mycelia growth of *Colletotrichum capsici*, significantly affecting fungal toxicity. The *Piper betle* L. crude extracts had the highest effectiveness (> 90%) against fungicides. The antifungal properties, like the *Piper retrofractum* Vahl., showed a significant inhibitory percentage (> 80%). Furthermore, hexane and ethyl acetate extracts of *Piper nigrum* L. and *Piper sarmentosum* Roxb. demonstrated excellent antifungal activity (> 80%). The phytochemical investigation of sample extracts revealed flavonoids, phenols, alkaloids, terpenoids, and steroids. As a result, the biological activity of plant extracts inhibited the growth of *Colletotrichum capsici* mycelia; they could serve as a source for the development of eco-friendly organic fungicides.

Keywords: antifungal, *Colletotrichum capsici*, chili, anthracnose, piper genus

INTRODUCTION

In the tropics, chili (*Capsicum annuum* L.) is considered an essential crop (May and Sang, 2016). Approximately 3.74 million hectares and 3.84 million tons of chili were cultivated on a global scale (FAO., 2020; Nicephore et al., 2021). Meanwhile, the harvest area in Thailand is approximately 5.50 thousand hectares with a yield of 2.84 million tons; the estimated revenue is $426.67 million (Chatsuda et al., 2021). However, causal fungal infections caused by *Colletotrichum* sp. pose the greatest threat to chili production from anthracnose disease (Raghavendra et al., 2020). Therefore, the infected chili was anthracnose, which caused worldwide losses of up to 50% (Po et al., 2008). This effect is responsible for both pre- and post-harvest chili damage. Fifty percent of cause losses have been reported in Malaysia (Wong et al., 2020), 10–54% in India, 20–80% in Vietnam (Raj et al., 2020), and up to 80% in Thailand (Manju et al., 2020). Six species of *Colletotrichum*, including *Colletotrichum capsici, C. siamense, C. acutatum, C. scovillei, C. asianum,* and *C. gloeoporioides*, have been linked to anthracnose in Indonesia, India, Korea, and Thailand (Than et al., 2008; May and Sang et al., 2016; Chatsuda et al., 2021). Chemicals, biological agents, or plant extracts could be used to control anthracnose disease (Manju et al., 2020). Chemicals have been used to prevent the disease anthracnose; the fungicide control was simple to manipulate and quick to respond. In contrast, long-term use of pathogenesis fungicide chemicals will result in human and environmental toxicity risks. Another option is natural plant products, which are essential sources of fungicide and are nontoxic to animals and the environment; these are regarded as environmentally friendly with excellent care. According to research, plant extracts and bio-control agents can combat anthracnose diseases in chili, such as inhibiting fungal growth with a 3% garlic bulb extract. *Acorus calamus* L., *Cymbopogon martinii* oil, and *Azadirachta indica* oil plant extracts were effective in inhibiting the growth of the anthracnose fungus (Po et al., 2008; May and Sang, 2016); *Azadirachta indica*, *Swietenia mahagoni*, and *Allium sativum* were the combination plant extracts that had a positive effect on disease reduction and crop yield in chili (Raj et al., 2020; Manju et al., 2020).

Piper retrofractum Vahl has literature on traditional medicines derived from various plant parts that treat anti-flatulent, antitussive, antioxidant, expectorant, antibacterial, anti-inflammatory, antimicrobial, and antifungal activities (Wan et al., 2020). This plant was evaluated for its efficacy against bacterial pathogens, including *Streptomyces albus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli,* and *Bacillus megaterium*, as well as the fungus *Aspergillus niger* (Mohib and Mustafa, 2007). In addition to exhibiting excellent

antibacterial activity, the extracts also demonstrated antifungal activity. Similarly, the bioactive methanol extract of *Piper retrofractum* Vahl was effective against pathogenic fungi, including *Fusarium moniliforme*, *F. oxysporum* DOAC2269, *Colletotrichum gloeosporioides* DOAC2213, and *C. acutatum* DOAC2285; the crude extracts demonstrated the potential for broad-spectrum antifungal activity in plant pathogens (Wattana, 2017). In addition, piperine showed significant antifungal activity against *Staphylococcus aureus* and *Bacillus subtilis* with MIC values of 225 g/mL (Wan et al., 2020). Lignans, sterol, alkaloids, flavones, tannin, and phenol were identified as antifungal constituents of *Piper retrofractum* Vahl.'s phytochemical constituents (Wan et al., 2020).

Piper nigrum L. contains an active component with diverse pharmacological properties, including antitumor, antioxidant, anti-inflammatory, antibacterial, insecticidal, and antifungal properties, among others (Nisar et al., 2012; Zoheir and Aftab, 2014). The antibacterial activity of piperine extract was demonstrated against *P. putida* and *Staphylococcus aureus*, with the highest inhibition observed in *Staphylococcus* (Krishna et al., 2019). Leaf extract with methanol has shown antimicrobial activity against *E. coli*, and leaf extract with ethanol is highly active against *S. aureus*; these extracts protect against dangerous pathogenic microorganisms (Mohd et al., 2014). The investigation of biologically significant phytochemicals, such as phenolics, flavonoids, alkaloids, terpenes, lignans, and steroids, could yield antifungal (Winda et al., 2021).

Piper betle L. was used to treat diseases in traditional medicine. This plant is believed to have bioactivity, including immunomodulatory, antifilarial, antileishmanial, antiamoebic, antioxidant, anti-inflammatory, antimicrobial, and antifungal properties (Biswajit et al., 2016). According to the research, there was a high activity level against the pathogens tested. For instance, *Colletotrichum capsici, Fusarium pallidoroseum*, *Botryodiplodia theobromae*, *Alternaria alternate*, *Penicillium citrinum*, *Phomopsis caricae-papayae*, and *A. niger* were inhibited significantly; the most minor inhibitory concentrations of ethanol extracts against these plant pathogens ranged from 0.01 mg/mL to 1 mg/mL (Kushagra et al., 2011). *Colletotrichum capsici* radial growth was most inhibited by leaf extract at a concentration of 10 µg/mL; methanol, chloroform, and acetone extracts inhibited growth by 85.25%, 78.53%, and 73.58%, respectively (Lucy et al., 2011). Researchers demonstrated the *in vitro* antifungal activity of hydroxychavicol isolated from *P. betle* L. against 124 fungal strains, including *Aspergillus* species (e.g., *A. flavus*, *A. parasiticus*, *A. niger*, and *A. fimigatus*) (Intzar et al., 2010). Tannins, essential oils, terpenoids, alkaloids, steroids, and phenol were among the phytochemical constituents extracted from *P. betle* L.; these bio-compounds could reduce the severity of fungus disease (Depi et al., 2020).

The pharmacological properties of *Piper sarmentosum* include anti-inflammatory, antioxidant, antimalarial, antimicrobial, antiprotozoal, antimicrobial, and antifungal properties (Rahman et al., 2016; Azelan et al., 2020). The IC_{50} values of the methanol extracts of *P. sarmentosum* against the fungi *P. fuscovaginae* and *Xanthomonas oryzae* were 10.42 and 24.69, respectively (Rahman et al., 2014). In addition, the essential oil exhibited high antifungal activity against *Rhizoctonia solani* and *Bipolarisoryzae* (Pragatsawat and Warinthorn, 2017). Moreover, at a concentration of 100 mg/mL, the aqueous extract inhibited the growth of *Fusarium verticillioides* with a diameter of 7.3 mm (Maizatul and Aiesyaa, 2020). *Piper sarmentosum* contains phytochemicals such as flavonoids, phenolic, saponins, terpenoids, steroids, tannins, and alkaloids; consequently, plant extracts are effective against fungi (Atefeh et al., 2013).

This study aimed to evaluate the efficacy of plant extracts in inhibiting *Colletotrichum capsici*, the agent responsible for chili anthracnose.

MATERIALS AND METHODS

Plant extracts

The experiment focuses on Piper genus plants collected in Thailand's Lampang province, including *Piper retrofractum* Vahl, *Piper nigrum* L., *Piper betle* L., and *Piper sarmentosum* Roxb. Plants were identified by the Biology Laboratory of Lampang Rajabhat University's Faculty of Science. The leaves were air-dried at room temperature, ground into a fine powder, and then successively percolated with n-hexane, ethyl acetate, and methanol (50 g × 0.3 L × 3 days three times) at room temperature, respectively. The filtrate solutions were evaporated under low pressure at 40 °C, and the resulting crude extracts were used to test bioactivity.

Phytochemical screening

The screenings of plant extracts utilize the method described by Trease and Evans (1989); testing methods reveal plants' chemical composition, such as phenols, flavonoids, alkaloids, terpenoids, and steroids (Rao et al., 2016).

Colletotrichum capsici material

Samples infect anthracnose disease with *Colletotrichum capsici* on chili fruit brought from regions Lampang province. The method used for separating and purifying fungi employs Tun et al.'s procedure (Tun et al., 2018).

Molecular variability

The identification of *Colletotrichum capsici* was based on a molecular analysis of DNA; using a modified version of Doyle and Doyle's Cetyl Trimethyl Ammonium Bromide technique, they were isolated from each pure culture. Under the following thermal settings, the ITS of rDNA was amplified by ITS3 (5′GCATCGATGAAGAACGCAGC3′) and ITS4 (5′TCCTCC GCTTATTGATATGC3′): 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 60 ºC on 30 s, and 72 ºC on 1 min. During PCR purification, the Gel/PCR DNA Fragments Extraction Kit (Geneaid), Taiwan, was utilized. The Macrogen, Inc. genetic analyzer was used to determine the sequences. Using the Basic Local Alignment Search Tool, sequences were compared to other sequences in the Gen Bank database (BLAST) (Meghana and Hiremath, 2019).

Evaluation of plant extracts inhibition Colletotrichum capsici in vitro

Evaluation of the efficacy of plant extracts against *Colletotrichum capsici* using the poisoned food diffusion technique. Four crude plant extracts were dissolved in lukewarm PDA and thoroughly mixed to produce a final 5, 10, and 20 mg/mL concentration. A procedure developed by Tun et al. (2018) was used to assess the antifungal activity of plant extracts (Tun et al., 2018). Fungi toxicity was reported as a percentage of inhibition and calculated according to the equation to be compared with the control.

$$P = 100 - \frac{100 \times R^2}{C^2}$$

P = Percentage of inhibitory activity against radial growth

C^2 = Radial growth of the fungal in the control

R^2 = Radial growth of the fungal in the treatment

Statistical analysis

All experiments were repeated five times. In all cases, analysis of variance (ANOVA) indicated that the data between the five repetitions were similar ($P > 0.05$). Thus, data of all variables from all five experiments were combined. Anthracnose control data obtained in all experiments of the extract concentration were used to estimate the effective extract concentration (%) to reduce colony diameter for each plant extract by two-way analysis of variance analysis, with the extract concentration as the independent variable and anthracnose as the dependent variable. The ANOVA (two-factor without replication) was used to compare all treatments' mean percentage of mycelial inhibition. The differences at $\alpha = 0.01$ were significant.

RESULTS AND DISCUSSION

Phytochemical screenings are shown in Table 1, including flavonoids, phenols, alkaloids, terpenoids, and steroids. Details the phytochemical evaluations of various plant extracts dissolved in solvents such as n-hexane, ethyl acetate, and methanol. Four plants were collected from a native plant for this experiment. This was research on using antifungal plant extract to treat chili diseases, except for *Piper nigrum* L. and *Piper sarmentosum* Roxb, which could not detect flavonoid compounds. Qualitative analyses of phytochemicals revealed the presence of steroids, alkaloids, and terpenoids in all plant extracts.

Table 1. Phytochemical screenings of plants

Plants	Flavonoid			Phenolic			Alkaloid			Terpenoid			Steroid		
	H	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**
P. retrofractum	−	+	−	+	−	−	+	−	+	+	+	+	+	+	+
P. nigrum L.	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+
P. betle L.	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+
P. sarmentosum	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+

H: n-hexane extracts, **E**: ethyl acetate extracts, **M**: methanol extracts, +: detected, −: non-detected

The plant extract exhibited a positive effect against the fungus *Colletotrichum capsici*, as indicated by the percentage inhibitions listed in Table 2. To advance potential new fungicides, it is necessary to investigate in vitro antifungal activity. Experiments with various solvents revealed that plant leaf extracts significantly ($P < 0.01$) inhibit mycelia growth. When the concentrations were 5, 10, and 20 mg/ml, respectively, the restraining was significantly enhanced (Fig. 1). The **H** extracts of *P. betle* L. demonstrated the greatest mycelia growth inhibition (95.76%). In the **H** extracts of *P. retroctum, P. nigrum* L., and *P. sarmentosum*, the percentage of mycelia growth inhibition efficacy was found to be 89.43, 85.75, and 76.15, respectively. Experiments with **E** extracts of *P. betle* L., *P. nigrum* L., *P. retrofractum*, and *P. sarmentosum* revealed that a concentration of 20 mg/mL significantly inhibited the pathogen by 95.36, 93.11, 90.66, and 87.38%, respectively. The **M** plant extracts inhibited the growth of *Colletotrichum capsici* effectively. They found that a concentration of 20 mg/mL inhibited the mycelia growth of *P. retrofractum, P. betle, P. Sarmentosum,* and *P. nigrum* by 95.57, 94.21, 68.05, and 2.47%, respectively.

Table 2. Inhibition of *Colletotrichum capsici* with plant extracts

Percentage of inhibition of *Colletotrichum capsici* with plant extracts										
Plant	**Con.** (mg/ml)	**H** MR ± SD (mm)		%	**E** MR ± SD (mm)		%	**M** MR ± SD (mm)		%
Control		50.69	±0.05	0	51.26	±0.47	0	48.51	±0.31	0
Pn	5	23.08	±1.00	79.28	19.63	±0.28	85.33	56.58	±0.44	No
	10	21.57	±0.27	81.89	16.76	±0.09	89.3	53.27	±0.16	No
	20	19.14	±0.34	85.75	13.45	±0.06	93.11	47.91	±0.61	2.47
Pr	5	21.83	±0.39	81.45	22.98	±0.09	79.9	14.31	±0.53	91.3
	10	19.14	±0.13	85.75	19.15	±0.57	86.04	12.24	±0.23	93.63
	20	16.48	±0.14	89.43	15.67	±0.07	90.66	10.21	±0.13	95.57
Ps	5	42.84	±0.14	28.57	31.66	±0.21	62.1	39.32	±0.08	34.29
	10	34.57	±0.48	53.48	27.07	±0.47	72.11	36.47	±0.12	43.48
	20	24.76	±0.75	76.15	18.21	±0.23	87.38	27.42	±0.46	68.05
Pb	5	13.65	±0.17	92.75	16.79	±0.57	89.27	28.29	±0.35	66.00
	10	11.1	±0.13	95.2	13.87	±0.50	92.68	20.71	±0.53	81.77
	20	10.44	±0.09	95.76	11.04	±0.26	95.36	11.67	±0.24	94.21

Each value represented the mean radial growth (MR) (5 replicates) ± standard deviation (SD); Significant differences ($P < 0.01$) are indicated by different alphabets. No = no inhibition; **Con**.: Concentration; **Pn**: *P. nigrum* L.; **Pr**: *Piper retrofractum* Vahl.; **Ps**: *P. Sarmentosum*; **Pb**: *P. betle* L.

This study revealed that crude leaf extracts from all four piper plants inhibited the growth of *Colletotrichum capsici* mycelia in a manner consistent with antifungal activity. Compared to previous research, the percentage of inhibition by crude plant extract of the Piper genus at 20 mg/mL was significantly higher when compared to the positive control of *Colletotrichum capsici.* According to research, extracts of the Piper genus demonstrated antibacterial and antifungal activities. Several flavonoids, phenols, alkaloids, terpenoids, and steroids were previously reported as phytochemicals from the Piper genus. This will be elaborated upon in the following section.

The number of active chemicals in an extract that can inhibit the growth of pathogens is shown to be inversely correlated with its concentration; specifically, the higher the extract concentration, the lower its ability to promote the growth of *Colletotrichum capsici*. Figure 1 shows a significant effect on the extracts tested. Extraction and concentration are greatly influential in inhibiting the growth of the pathogen *Colletotrichum capsici*. The higher the concentration of the extract, the higher the percentage of inhibition, and the growth of mycelium on PDA media decreases to an inhibition zone which feat *Colletotrichum capsici* pathogens hard to grow due to exposure to active fungicides in the extract. The leaf of *P. betle* L. has anti-fungal properties with essential oil components, namely eugenol, acetyl eugenol, and hydroxychavicol compounds (Intzar et al., 2010) that can inhibit pathogen growth (Kushagra et al., 2011; Depi et al., 2020). *Piper retrofractum* Vahl., showed

demonstrated antifungal activity. Similarly, the bioactive extract of *P. nigrum* L. (Krishna et al., 2019) and *P. sarmentosum* (Maizatul and Aiesyaa, 2020) were effective in inhibiting anthracnose (Wattana, 2017). Extracts have antifungal properties because they are found to contain active components; piperine, β-caryophyllen, limonine, eugenol, acetyl eugenol, and hydrocinnamic acid compounds, consequently could effectively inhibit the growth of fungal mycelium (Atefeh et al., 2013; Wan et al., 2020; Winda et al., 2021). The test results are reasonably compatible with earlier research reports for all experimental plants. Therefore, the crude extracts have the potential for antifungal activity in plant pathogens.

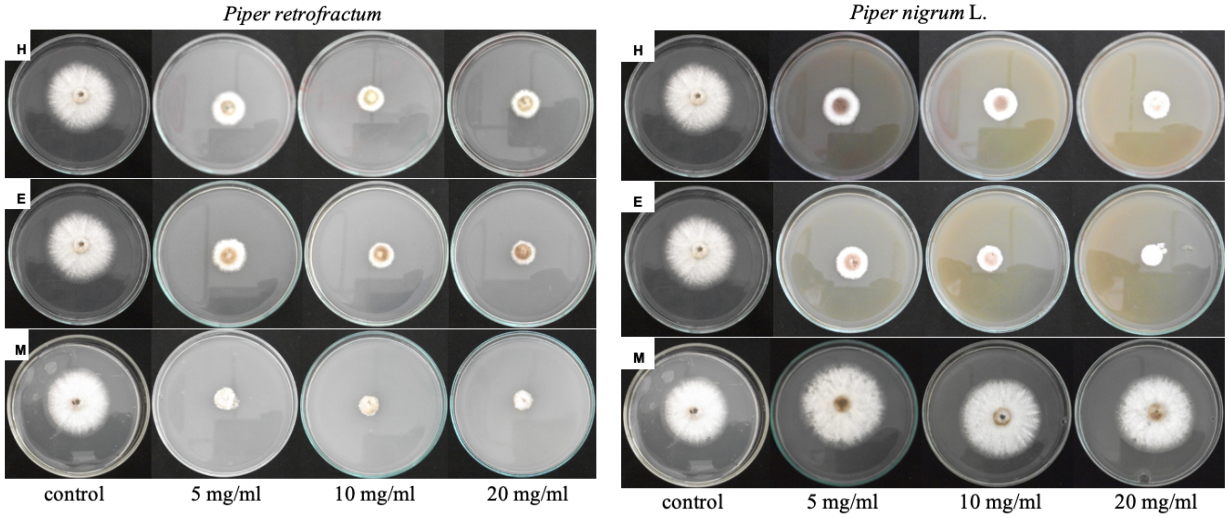

Figure 1. Inhibition of *Colletotrichum capsici* with *Piper* genus (in PDA).

CONCLUSIONS

This study demonstrates the highest effectiveness against the chili-causing fungus *Colletotrichum capsici*. Extracted crude plants revealed chemical components, including flavonoids, phenols, alkaloids, terpenoids, and steroids. Utilizing natural product plants as fungicides in organic agriculture has the potential to benefit from the results of this study. This study revealed that bioactive metabolites from plants have the highest potential as fungicides for agriculture and will contribute to the future management of these essential diseases.

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