ORIGINAL PAPER

Evaluation of Antifungal activity against *Pyricularia oryzae*, the cause of Rice Blast Disease, from the extract of *Piper spp.*

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Abstract. This research was finding out that bioactivities of piper genus extracts were inhibiting growth of *Pyricularia oryzae*, the cause of rice blast disease. The crude extract of four species including *Piper retrofractum* Vahl, *Piper betle* L., *Piper sarmentosum* Roxb., and *Piper nigrum* L., were screened for their potentail antifungal activity under *in vitro* method. The percolation techniques were performed by using hexane, ethyl acetate and methanol as extraction solvents, respectively. Then, 10, 30 and 50 mg/ml of samples were tested *in vitro* by the poisoned food technique. Our results revealed that our extracts consists of some phytochemicals including alkaloids, flavonoids, phenols, steroids, and terpenoids. The higher concentration of extracts, the more ability to inhibit mycelial growth of *P. oryzae*. The highest activity was observed from the ethyl acetate extract of *P. betle L.* which completely inhibited the fungus (100%). Several extracts revealed high antfugal activity (>90%) such as the hexane extract of *P. betle* L, *P. sarmentosum* Roxb. and *P. nigrum* L., the ethyl acetate extract of *P. retrofractum* Vahl., *P. betle* L. and *P. sarmentosum* Roxb., and methanol extract of *P. betle* L. In conclusion, the extracts of piper genus could be applied as a potential alternative of safe agricultural fungicide, especially, against *P. oryzae*.

Keywords: Piper, Antifungal, Rice blast, *Pyricularia oryzae*

1. Introduction

Rice is an important food crop and is consumed by more than 50% of the world's population; in addition, it's an essential food for humankind (Yin et al. 2021, Tripathi et al. 2011). Rice yields are decreasing as a result of climate change; tropical humid climates in Asia are also contributing significantly to the pestilence of rice blast disease in Thailand (Kiguchi et al., 2021). The fungal *Pyricularia oryzae* causes rice blast disease; rice blast disease is a destructive disease that can cause up to 30% product loss. The report on rice blast fungus, a widespread disease in Thailand, found 59 strains of *P. oryzae*, the cause of the damage to rice (Longya et al. 2020). The farmers must manage rice blast disease control with chemical and biological methods etc. (Persaud et al. 2019, Agbowuro et al. 2020), but favor requires using chemical control because of the easy and quick response. Long-term use of chemical fungicides, on the other hand, poses a high risk to the environment, humans, and the development of fungicide resistance, as it causes the fungus to induce new pathogens (Flora et al. 2012; Panwey et al. 2018). The research has studied and reported the anti-fungal properties of plant extracts to control *P. oryzae* fungal growth as an alternative to using chemical fungicides. Plants are important sources of antifungal compounds, with few toxicity effects on mammals and the environment and low bioaccumulation due to their ease of decomposition; botanical fungicides are also environmentally friendly.

The reported antifungal properties in plants have phytochemically efficient fungicides such as phenol, tannin, terpenoid, alkaloid, quinone, flavonoid, and xanthone, etc. (Suprapta et al. 2014). Plants have been well-known for their research reported fungicides, such as the crude extracts of *Agave sisalana* and *Cymbopogon citrates* indicated inhibitory effect on the growth and spore of *P. oryzae* (Kassankogno et al. 2015); the leaf extract of *P. caninum* exhibited a high inhibitory activity against *P. oryzae* (Suriani et al. 2015), and the *Piper caninum* a highest inhibition of 90.5% the fungal *P. oryzae* (Suriani. 2018). The research focus of the study was to evaluate the efficacy of plant extracts in inhibiting *P. oryzae in vitro* in rice blast disease.

Piper retrofractum Vahl. has been used as an ancient medicine and has a number of biological activities including antitussive, antioxidant, antiobesity activity, antioxidant, antibacterial, and antifungal properties (Ratwatthananon et al. 2020). Specifically, the efficiency of essential oils of *P. retrofractum* from fresh leaves was active against pathogenic bacteria, namely *S. aureus*, *M. luteus*, and *B. subtilis*, with clear zones of 8.0, 8.5, and 9.7 mm, respectively (Jamal et al. 2013). Including, the fresh fruits of *P. retrofractum*, they were extracted with solvents such as ethanol, methanol, isopropanol, dichloromethane, water, hexane, and methanol/isopropanol, which showed efficacy against plant disease causing fungi: *F. moniliforme* DOAC 1224, *F. oxysporum* DOAC 2269, *Colletotrichum gloeosporioides* DOAC 2213, and *C. acutatum* DOAC 2285. The isopropanol extract showed the highest inhibition zone diameters against *C.gloeosporioides*, *F. oxysporum*, *F. moniliforme* and *C. acutatum*, with a clear zone of 5-11 mm (Panphut et al. 2017). Interestingly, this plant had presented insecticidal activities against different insects for example *Spodoptera litura* Fab., *Spodoptera exigua* Hubner, *Plutella xylostella* L., *Blattella germanica* L. and *Culex quinquefasciatus* Say (Ratwatthananon et al. 2020). Furthermore, the *P. retrofractum* extracted by methanol, hexane, dichloromethane, isopropanol, and acetonitrile showed activity against 10 pathogenic creatures that opportunistic infections including *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC2921, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* TISTR1843, *Pseudomonas aeruginosa* ATCC741, *Salmonella typhi*, *Vibrio parahaemolyticus*, and *Candida albicans* ATCC90020. Moreover, the methanol extract showed the highest antibacterial activities than other solvents with inhibition zone diameters from 0.5 to 8.0 mm, respectively (Panphut et al. 2020). The antibacterial activities of the *P. retrofractum* isolated phytochemicals were in addition evaluated used in the micro-dilution method; piperine show the significant activities against *S. aureus* and *B. subtilis* had MIC values of 225 µg/mL (Salleh et al. 2020). Phytochemicals of *P. retrofractum* were presented as chemical constituents from fruit extract with different solvents (methanol, ethyl acetate and *n*-hexane). They included quinine, sterol, alkaloids, flavones, tannin, and phenol, which supported their function for bioactivity purposes (Jadid et al. 2018, Salleh et al. 2020).

Piper betle L. had several bioactivities and was used in traditional medicine. Much research concern *P. betle* L. have reported that its considerable phytochemical constituents and bioactivity properties for instance anti-malaria, anticancer, anti-filarial, anti-allergic, antioxidant, antibacterial and antifungal etc. (Patra et al.

2016). In reported research, the leaf extracts were composed of essential oil and phenolic constituents with indicative antimicrobial properties against oral bacteria and pathogens, e.g., *Vibrio choleraeogawa*, *Diplococcus pneumoniae* and *Klebsiella aerogenes* (Ramji et al. 2002). Subsequently, studies show the bioactive compounds isolated from this plant have antimicrobial activity against pathogenic bacteria were sterol constituents (Mula et al. 2008). Afterwards, research presented the *in vitro* antifungal activity of hydroxychavicol isolated from the leaf of *P. betle* L., getting involved on 124 fungal strains: together with *Aspergillus* spp. e.g., *A. flavus*, *A. parasiticus*, *A. niger* and *A. fumigatus* (Sharma et al. 2009). Similarly crude extracts of leaf extraction from *P. betle* L. with ethanol were tested *in vitro* antifungal activity, showing 100 percentage inhibitions against plant pathogenic fungi (Singburaudom et al. 2015). Recently, phytochemical constituents consisting of essential oils, terpenoids, and phenol, were obtained from the blends of *P. caninum* and *P. betle*var. Nigra leaves extracts, these bio-compounds could reduce the intensity of blast fungus disease, *P. oryzae* (Suriani et al. 2020).

Piper sarmentosum was a scientific finding in addition to proving different pharmacological actions of various parts of *P. sarmentosum*, such as antituberculosis, anti-inflammatory, antioxidant, antimicrobial, antifeedant, antimicrobial, and antifungal activities (Seyyedan et al. 2013). For example, the methanol extracts of native Malaysian plants, which *P. sarmentosum* showed against fungal *Aspergillus flavus*, *Microsporum canis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Candida albicans* (Nazmul et al. 2011). In Thai medicinal teas were ethanol extracts presented *P. sarmentosum* have antifungal activity, *Aspergillus fumigates* and *C. albicans* (Cheeptham et al. 2002). Furthermore, the first report of myristicin for anti-mycelia activity against rice pathogenic fungal (Pauli et al. 2010). Similarly, the sarmentine and brachyamide B were appraised for antibacterial activity also the inhibition of RNA formation affecting mycelia growth (Rukachaisirikul et al. 2004, Mueller et al. 2008, Hussain et al. 2012). Constituents of myristicin and brachyamide B were interesting and showed higher capability anti-rice pathogens activity (Lee et al. 2014, Chanprapai et al. 2017).

Piper nigrum L. bioactive constituent presented several pharmacological activities for example antioxidant, anti-inflammatory, antibacterial, insecticidal, and antifungal, etc. (Damanhori et al. 2014, Joshi et al. 2018). The essential oil and oleoresin were extracted from *P. nigrum*, *C. sativum*, and *C. domestica* for this effect on *P. oryzae* in rice. This showed that pepper oil was most efficient against a fungal pathogen; it decreased the infection frequency in *P. oryzae* (Sukanya et al. 2011). While *P. nigrum* was tested for activity against *A. niger*, *A. alternate*, *A. flavus*, *B. subtilis*, *E. coli*, *S. aureus*, *P. aeruginosa* and *F. oxysporum*; showed the highest activity against gram positive *S. aureus* and gram negative *E. coli*. Furthermore, piperine showed the highest activity against *F. oxysporum* (Rani et al. 2013). Seeds extracted with hexane and ethanol showed explicit leishmanicidal activities against *Leishmania donovani* promastigotes and amastigotes *via* apoptosis (Chouhan et al. 2015). Meanwhile, extraction of *P. nigrum* with solvents namely carbon tetrachloride, benzene, ethyl acetate, acetone, methanol, ethanol, and distilled water were tested activities against gram-positive and gram-negative bacteria e.g., *S. albus*, *S. typhi*,

E. coli, *B. megaterium*, and fungus, *P. aeruginosa* (Sapam et al. 2018). Then, *P. nigrum* extract inhibited the *in vitro* mycelia growth of *M. Oryzae* (70.68%) and difenoconazole showed activities inhibiting mycelia growth percentage (83.55%) (Aslam et al. 2019). The researchers isolated various types of compounds such as phenolics, flavonoids, alkaloids, terpenoids and steroids; they were for that reason a potential source for fungicides (Ahmad et al. 2012).

2. Materials and Methods

2.1 Plant extracts

Samples for this study, namely *P. retrofractum* Vahl, *P. betle* L., *P. sarmentosum* Roxb., and *P. nigrum* L., were collected from Lampang province, Thailand. Plant samples were identified by the Laboratory of Science Program in Biology, Department of Biology, Faculty of Science, Lampang Rajabhat University, Lampang, Thailand. Plant identification was accomplished by comparing known herbarium specimens with illustrations, photographs, and literature in an herb database. The leaves were air dried at room temperature; after that, all were powdered using a blender. The leaves powder was consecutively percolated with solvent; hexane, ethyl acetate and methanol (100 g ×0.5 L × 3 days × 3 times), at room temperature, afterwards filtration respectively. The filtrates were evaporated with an evaporator under low pressure at 40 ˚C, to get the crude extracts being used to test inhibiting fungal activity.

2.2 Phytochemical screening

The phytochemical screening of the sample extracts was done using the applied method of Trease and Evans, for the investigation of phenols, flavonoids, alkaloids, terpenoids, and steroids for all the samples (Rao et al. 2016).

2.2.1 Test for phenols

Sample extracts 0.10 g dissolve with ethanol 1.0 mL in a test tube, mixes 1.0 mL of water and then adds two drops of $FeCl_3$ solution. It was indicated blue, green, red, or purple colorable are positive tests.

2.2.2 Test for flavonoids

Sample extracts 0.10 g dissolve 5.0 mL by solvent, then add 1-5 drops of concentrated HCl and 0.50 g magnesium turnings. These are flavonoids that have been discovered and have a pink or red color.

2.2.3 Test for alkaloids

The solvent 2.0 mL taken in a test tube of sample extracts 0.10 g and then adds 2N HCl 0.20 mL, followed by 1.0 mL of Meyer's reagent. Result alkaloids positive tests appear a yellowish color.

2.2.4 Test for terpenoids

Sample extracts 0.10 g put in a test tube, after adding 2.0 mL of chloroform; finally mix with 3 mL conc. H_2SO_4. Result positive tests show a layer, reddish brown color of the interface.

2.2.5 Test for steroids

The sample 0.10 g dissolves with 3.0 mL acetic anhydride, after that drops of conc. H_2SO_4. They were appearances of bluish green color indicating a positive test.

2.3 Rice blast material

Rice leaves infected with *P. oryzae* are collected from Lampang province's growing regions. Fresh leaves were surface sterilized with ethanol and after that use 2% sodium hypochlorite cleaning. Following, these pieces were cleaned with sterilized water and placed onto potato dextrose agar plate incubation. Incubation at 37 ˚C take 24-hour, conidio-spores from the surface of the lesion were spread onto water agar and incubated. Germinated conidio-spores were isolated and

transferred onto potato dextrose agar. The fungal were sub-cultured to obtain pure cultures and store them for further testing (Tun et al. 2018).

2.4 Molecular variability

The molecular characterization of genomic DNA sequences was extracted from mono-conidiary cultures of *P. oryzae* using a modified version of the Cetyl Trimethyl Ammonium Bromide method of Doyle (Doyle et al. 1897). ITS regions of the isolated *P. oryzae* isolates were amplified using primers ITS3 (5´GCATCGATGAAGAACGCAGC3´) and ITS4 (5´TCCTCC GCTTATTGATATGC3´). The sequence analysis was compared with the sequences in the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (Meghana et al. 2019).

2.5 Evaluation of plant extracts against P. oryzae in vitro

The poisoned food method was used to evaluate the efficacy of sample extracts in inhibiting *P. oryzae*. Sample extracts were thoroughly mixed in the necessary warm potato dextrose agar to achieve a final concentration of 10, 30, and 50 mg/mL. A completely randomized design with three replicates per treatment was adopted, and 20 mL of such media was poured in each sterilized Petri plate. When solidification, fungal cultures of *P. oryzae* (1×10^8 spores/mL) in discs were punched off the 7-day-old at the center of the Petri plate. The plates were incubated at room temperature in the Biological Oxygen Demand incubator and observed for radial growth every 24 hour after inoculation; until the entire surface of the control Petri plates was covered with mycelium growth. The growth of mycelium inside Petri plates improved with different concentrations, and sample extracts were measured and compared with the control. Fungal toxicities were reported in terms of percentage inhibition and calculated according to the formula (Tun et al. 2018).

$$PIA = \frac{Rc - Rt}{Rc} \times 100$$

PIA = Percentage of inhibitory activity against radial growth

Rc= Radial growth of the fungal in the control

Rt= Radial growth of the fungal in the treatment

3. Results

The study of phenols, flavonoids, alkaloids, terpenoids, and steroids was reported in research.The phytochemical tests of sample extracts using different solvents such as hexane, ethyl acetate, and methanol produced results shown in Table 1. In this research, four plants were collected from native plants in Lampang. Qualitative determinations of phytochemicals were indicative of alkaloids, steroids, and terpenoids in the sample extracts. Unlike the other tested sample plants, no flavonoids were found in sample extracts of *P. sarmentosum* Roxb and *P. nigrum* L.

Table 1. Qualitative screening of phytochemicals in sample extracts

Plants/Test	Flavonoid			Phenols			Alkaloids			Terpenoid			Steroids		
	H	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**	**H**	**E**	**M**
Piper retrofractum Vahl	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+
Piper betle L.	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
Piper sarmentosum Roxb.	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
Piper nigrum L.	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+

H: Crude hexane extract, **E**: Crude ethyl acetate extract, **M**: Crude methanol extract
(+) indicates the presence, (-) signifies absence

Table 2. Evaluation of the inhibition of *P. oryzae* by sample extracts

Plants	% Inhibition of *P. oryzae* with sample extracts								
	H (mg/ml)			**E** (mg/ml)			**M** (mg/ml)		
	10	30	50	10	30	50	10	30	50
Piper retrofractum Vahl	63.73	63.87	88.82	69.33	69.66	94.09	15.47	46.27	62.41
Piper betle L.	90.72	91.09	97.81	100	100	100	38.79	83.91	97.12
Piper sarmentosum Roxb.	92.70	93.88	94.87	89.01	92.14	93.08	37.46	48.68	56.19
Piper nigrum L.	89.24	89.81	90.63	82.59	84.94	87.81	53.99	59.30	61.50

H: Crude hexane extract, **E**: Crude Ethyl acetate extract, **M**: Crude methanol extract
Each value represented the mean (3 replicates); Standard Deviation (SD<0.50).

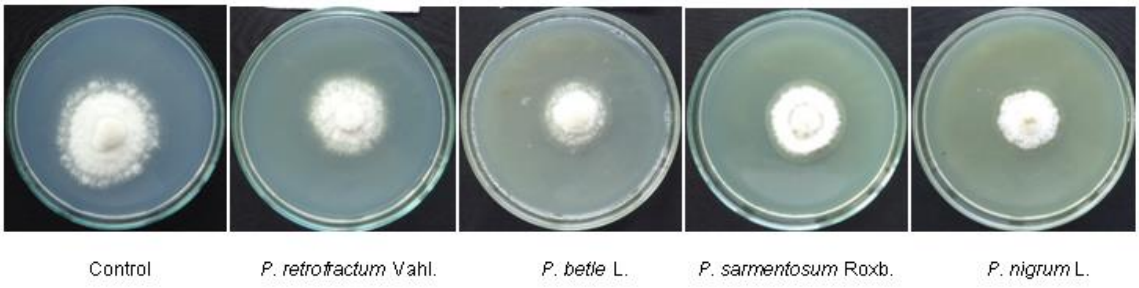

Figure 1. Inhibition of *P. oryzae* with crude Ethyl acetate extract of plants (in PDA).

The efficacies of plant extracts bioactivity to *P. oryzae* were evaluated by the percentage inhibitions shown in Table 2. Values, which accurately indicate the percentages of inhibitory activity against effective control, were correctly calculated from mean values. The study of *in vitro* antifungal activity is important and brings about the development of new potential fungicides. The intensity of mycelial growth was significantly reduced (P 0.05) after treatment with different sample extracts. All the tests of the sample hexane extracts showed inhibition of mycelial growth of *P. oryzae*. The restraining effect increased following the concentrations of 10, 30, and 50 mg/mL, respectively. Treatment with **H** extracts of *P. betle* L., *P. sarmentosum* Roxb., *P. nigrum* L., and *P. retrofractum* Vahl.; at 50 mg/mL indicated significantly high inhibiting of the pathogen percentages of 97.81, 94.87, 90.63, and 88.82, respectively (Figure 1). The tested **E** extracts of *P. betle* L. gave the highest mycelia growth inhibition (100%); while the high mycelia growth inhibition effect percentages of 94.09, 93.08, and 87.81 were found in the **E** extracts of *P. retrofractum* Vahl, *P. sarmentosum* Roxb., and *P. nigrum*

L., respectively. The differences in **M** extracts of plants have an inhibitory effect on *P. oryzae* growth; where treatment with extracts at 50 mg/mL inhibited the mycelia growth percentage, they were 97.12, 62.41, and 61.50 in the extracts of *P. betle* L., *P. retrofractum* Vahl, and *P. nigrum* L., respectively.

4. Discussion

This research revealed that sample extracts of four *Piper* genus plants were able to inhibit suitable fungal activities, all in against the mycelial growth of *P. oryzae*. Compared with the positive control of *P. oryzae* isolated appear the percentage of inhibition by crude sample extract of four piper at 50 mg/mL concentration exhibited higher against previous research report. Research reported on *Piper spp.* extracts showed antibacterial and antifungal activity. Previously reported phytochemicals from the *Piper* genus includedseveral compounds such as flavonoids, phenols, alkaloids, terpenoids, and steroids. This will be discussed in detail as follows.

The *P. betle* L. showed the effective inhibitors, which included a certain indication that the crude extract contained active components that had antifungal properties. The plants contain phenolic compounds such as hydroxychavicol (Singburaudom et al. 2015), cavicol, cavibetol, carvacrol, eugenol, and allylpyrocatechol (Sharma et al. 2009, Suriani et al. 2020). These active compounds are reasonably assumed to inhibit fungi. This study also demonstrated that it could be effective against pathogenic fungi in rice. The compounds reported from the extracts of *P. sarmentosum* Roxb. were identified as isocaryophyllene, myristicin, *β*-asarone, *α*-asarone, apiol, 1,1-dichlorocyclopentane, and 14-chloro-1-tetradecanol (Jee et al. 2004, Maizatul et al. 2020). Myristicin, the aromatic ring of phenylpropanoids was reported to possess influential antibacterial activity against rice pathogenic fungi (Chanprapai et al. 2017). *P. nigrum* L. naturally contains a bioactive compound known as piperine. Among the alkamides isolated from *P. nigrum* L., piperine, piperettine, and piperettyline have been reported to have efficient antimicrobial properties (Chitra et al. 2013). The potential efficacy of leaf extracts of *P. retrofractum* Vahl for controlling the growth of important phytopathogenic fungi was evaluated. The compounds were identified: piperine, α-humulene, caryophyllene oxide, viridiflorol, globulol, *β*-selinene, spathulenol, (*E*)-nerolidol, linalool, and 3-pentanol were the major compounds (Rahman et al. 2011).

This research shows the phytochemical and bioactive properties of crude extracts of plants. The plants use organic solvents, such as hexane, ethyl acetate, and methanol for extraction. The study of bioactive metabolites extracted from plants shows that they are effective against rice disease-causing fungal *Pyricularia oryzae* and the crude plant extracts were detected; they contained phytochemicals such as alkaloids, flavonoids, phenols, steroids, and terpenoids, which are consistent with previous reports. The results show potential for using extracted plants as fungicides in organic farming. This research indicates a bioactive metabolite from *the Piper spp.* shows the highest potential as a fungicide product for rice agriculture in the future.

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