Journal of Engineering and Applied Sciences 12 (23): 7321-7324, 2017
ISSN: 1816-949X
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Anti Plant Pathogen of Bioactive Metabolite from *Piper retrofractum* Vahl

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Abstract: Plant pathology anthracnose by *C. acutatum* and *C. gloeosporioides* and root rot and vascular wilt by *F. oxysporum* and *F. moniliforme* caused a serious problem of crop loss in Thailand. Agricultural chemicals such as fungicides were sprayed on crops to control diseases that damaged field crops. This has created more problems because fungicide residues have been found on food for human consumption, mostly from post-harvest treatments. *Piper retrofractum* Vahl (long pepper) is in the Piperaceae family which can be found in Kanchanaburi, Ratchaburi and Chanthaburi Provinces of Thailand. It has been used as a Thai traditional herb ingredient in various treatment formulas. The aim of this research is to extract metabolite from long pepper using several solvents and to perform therapeutic efficacy testing against fungal of plants pathogen disease. The results showed that the bioactive metabolites extracted from *P. retrofractum* are effective against plant disease-causing fungi *Fusarium moniliforme* DOAC 1224 causes the stalk rod disease, *F. oxysporum* DOAC 2269 causes the yellow wilt disease, *Colletotrichum gloeosporioides* DOAC 2213 and *C. acutatum* DOAC 2285 both cause the most destructive fungal disease, anthracnose. Total of crude extracted found in isopropanol is 2.193±0.579 g/100 g of fresh pepper. Agar disc diffusion assay performed crude extracted concentrations 713 mg/mL against *C. gloeosporioides* is clear zone 5.25±0.35 mm. Against *C. acutatum* is clear zone 11.00±4.24 mm, against *F. moniliforme* is clear zone 9.00±4.24 mm and against *F. oxysporum* is clear zone at 6.50±0.71 mm. Moreover, the results can be concluded that isopropanol is an effective solvent for extracting bioactive compounds from fresh *P. retrofractum*. The crude metabolites have shown potential for broad range antifungal activities in plant pathogens.

Key words: Antifungul, bioactive metabolite, extraction, *Piper retrofractum,* plant pathogenic fungi, potential

INTRODUCTION

Pant pathogenesis caused by fungal infection is one of the problems of crop losses in Thailand. *Fusarium moniliforme* has been reported to cause root rot in corn, maize, wheat and potatoes. *F. oxysporum* causes not only the crown rot and root rot but also yellow wilt in many plants such as tomato, chili, legumes and sugarcane. *Fusarium* species are the group of Deuteromycetous fungi that can be found commonly in the soil. *F. oxysporum* can produce mycotoxin a substance which can be accumulated in plant tissues such as those in tubers and subsequently passed into animals and humans upon the consumption (Gupta *et al*., 1991). The most common mycotoxins produced by toxigenic Fusaria include fumonisin, deoxynivalenol and zearalenone. All contaminate maize grains and therefore, represent similar health hazard like aflatoxins (Pechanova and Pechan, 2015). *F. oxysporum* and *F. moniliforme* cause troubling symptoms associated with vascular wilt disease in the host plant which generally include discoloration of the vessels, wilting, defoliation, stunting and plant death (Sain and Rep, 2015). *Colletotrichum* species are a group of ascomycete fungi that are plant pathogens causing anthracnose disease. These fungi can infect a wide range of host plant species (Agrios, 2005). *C. gloeosporioides* has widely been associated with a post-harvest problem in fruits such as citrus, apple, olive, mango, banana and strawberries (Hyde *et al*., 2009). Conidia of *C. gloeosporioides* germinate on the surface of the fruit, forming dark rown to black spots which become sunken lesions on the rind tissues. Symptoms of anthracnose such as sunken on leaves, stems, flowers and tips. Papaya lesions can be found on leaves, stems, flowers and tips. Papaya and mango infected with anthracnose exhibit round, water soaked and sunken spots on the body of their ripening fruits (Nelson, 2008). These fruits produced may be infected by the disease since they were young but developed symptoms later especially during and after ripening of the infected, mature fruit. *C. acutatum* is a major anthracnose in plant stem and leave. Symptoms include tissue necrosis, corm rot, leaf crinkles and characteristic spiral twisting of floral peduncles. The pathogen, diagnosed as *C. acutatum*, causes typical anthracnose symptoms as well as root necrosis and stunting of affected plants (Freeman *et al*., 2000).

Long pepper or *Piper retrofractum* Vahl is a flowering vine in the family Piperaceae, cultivated for its fruit. The dried fruit has long been used as a spice and seasoning. Thailand is located in tropical humidity zone which provides the best condition for growing *P. retrofractum*, especially, in the central part of Kanchanaburi, Ratchaburi, Phetchaburi and Chanthaburi Provinces. Locals use *P. retrofractum* fruit as a key ingredient in various recipes of their traditional medicine. There has been no report of toxicity from long pepper. Fruit of long pepper was analyzed for its chemical profile. Extracted bioactive metabolites from *P. retrofractum* were found to contain alkaloid component amide derivative with anti-flatulent, expectorant, antitussive, antifungal and appetizing properties. In addition, it was reported to possess gastroprotective and cholesterol-lowering properties which could be useful for application in traditional medicine (Kim *et al*., 2011; Muharini *et al*., 2015; Barceloux, 2009). The aim of this research is to extract metabolite from long pepper using several solvents and to perform therapeutic efficacy testing against fungal of plants pathogen disease.

MATERIALS AND METHODS

Fungal cultures: *Fusarium moniliforme* DOAC 1224, *Fusarium oxysporum* DOAC 2269, *Colletotrichum gloeosporioides* DOAC 2213 and *Colletotrichum acutatum* DOAC 2285 were obtained from Plant Protection Research and Development Office, Department of Agriculture Thailand. There were grown on PDA at 30°C for 10-15 days depending on conidia formation.

Plant extract sample: Fresh fruits of *Piper retrofractum* Vahl (long pepper) obtained from Kanchanaburi orchard, Thailand were ground in a blender. Then, hydrophilic compounds were extracted using polar solvents such as methanol, ethanol or isopropanol. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in a ratio of 1:1 was used. Then, the crude extract was removed out of the solvent by a rotary evaporator (Rotavapor® R-300, BUCHI) and was left dry in the desiccator over silica gel. The crude continued to weigh and dissolved by DMSO (Dimethyl Sulfoxide) for the next experiment.

Antifungal disc diffusion assay: Organisms were subcultures on Potato Dextrose Agar (PDA) at 30°C for 15 days until the spore production. Following growth, conidia were harvested using a 0.1% Tween 80 (v/v) solution in sterile saline and a number of spore forming was counted using a hemocytometer. The conidial suspension was adjusted to 1.0×10^6 conidia/mL. PDA plates were streaked evenly with a swab dipped into the standardized inoculum suspension. Lids were left ajar for 3 min in a laminar flow cabinet to allow any excessive surface moisture to be absorbed into the agar before the drug-impregnated disks were applied. Paper disks 6 mm diameter containing the crude solution 20 μL were dried and then applied to the surfaces of inoculated plates. Plates were incubated at 30°C for 4-7 days to allow fungal growth. Inhibition Zone Diameters (IZD) were measured in millimeters (Nweze *et al*., 2010). To evaluate the reproducibility of our method, a new inoculum was prepared for each replicate, all isolates were run in triplicate and the standard deviations were determined.

RESULTS AND DISCUSSION

Fungal cultures: The conidia were produced on SDA 30°C after 14 days and observed by slide culture technique, then dyed with lactophenol cotton blue. Both of plant pathogenic fungi *C. acutatum*, *C. gloeosporioides*, *Fusarium oxysporum* and *F. moniliforme* are conidiospore forming. The conidia of *C. acutatum* under microscopic were germinate tip hyphal produced in chains of round shape and rough surface conidia. *C. gloeosporioides* are two types of conidiospore forming club shape bilateral crosswise septate conidia and appresoria formation with oval shape dark-brown colour. We found significant differences among isolates in appresoria shape in *C. gloeosporioides* DOAC 2213 average shape of 70-80 μm. *F. oxysporum* (DOAC 2269) produced two types of conidia which are macro conidia lemon shape 70-80 μm and fusiform. There were phialides development and conidiation from the hypha apex. Another *F. oxysporum* DOAC 2269 conidia are fusiform formation oval to club-shaped with the basal cell. *F. moniliforme* DOAC 1224 produced two types of conidia which are small club shape and large club shape.

Disc diffusion antifungal sensitivity testing: Development of disk diffusion assay for determining the antifungal susceptibility of plant pathogenic fungi was modified from Nweze *et al*. (2010). A filter-paper disk, impregnated with the *P. retrofractum* Vahl extracted compound to be tested is placed on the surface of the agar. The bioactive compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disk and will decrease as a distance from the disk increases. If the compound is effective against plant pathogenic fungi at a certain concentration, no mycelium colonies will grow where the concentration in the agar is greater than or equal to the

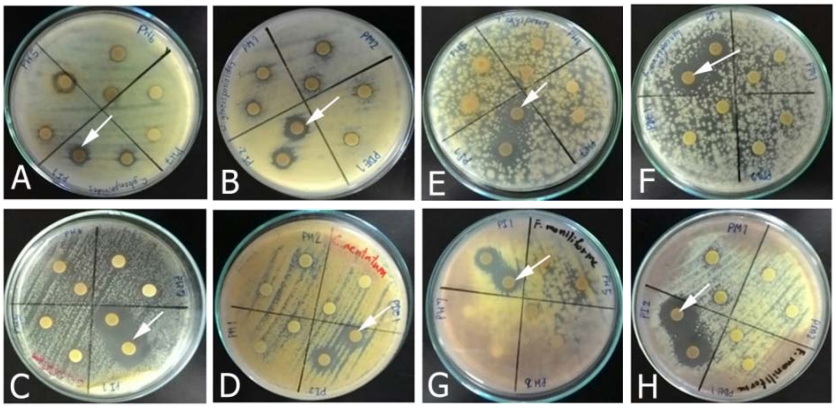

Fig. 1: Inhibition zone metabolite extract *P. retrofractum* Vahl against plant pathogenic fungi; A and B: Isopropanol crude extract anti *C. acutatum* DOAC 2285 (arrow), C and D: Isopropanol crude extract anti *C. gloeosporioides* DOAC 2213 (arrow), E and F: Isopropanol crude extract anti *F. oxysporum* DOAC 2269 (arrow), G and H isopropanol crude extract anti *F. moniliforme* DOAC 1224 (arrow)

Table 1: The results of antifungal activity of crude extract fruit of *P. retrofractum* Vahl

		Antifungal clear zone (mm)			
Crude extract	Concentration (mg/mL)	Anti *C. gloeosporioides*	Anti *C. acutatum*	Anti *F. moniliforme*	Anti *F. oxysporum*
Ethanol	447±1.2	1.34±0.87	1.43±1.31	1.66±0.93	0.43±1.60
Methanol	339±0.3	2.00±0.00	0.75±0.00	1.00±1.41	0
Isopropanol	713±0.6	5.25±0.35	11±4.24	9.00±4.24	6.5±0.71
Dichloromethane	346±1.1	2.68±1.08	1.52±1.56	1.73±1.90	0.82±1.83
Water	223±1.3	0.79±0.64	0	0	0
Hexane	63±0.1	1.67±0.29	0	2.00±1.80	0
Methanol/Isopropanol	579±1.3	2.63±0.75	10.25±0.11	8.38±1.70	2.75±5.50
Dichloromethane/Ethanol	336±1.1	1.69±0.63	2.23±1.66	2.79±2.20	0

effective concentration. This is the zone of inhibition and Inhibition Zone Diameters (IZD) were measured (Fig. 1). Eight Solvents used for *P. retrofractum* Vahl extraction were ethanol, methanol, isopropanol, dichloromethane, water, hexane, methanol/isopropanol (1:1) and dichloromethane/ethanol (1:1) (Table 1). The result revealed the highest extraction yield by the solvent isopropanol which was 2.193±0.579 g/100 g of fresh fruit long pepper. Disc diffusion antifungal four isolates were performed and compared IZD between eight types of crude extracted metabolite represented in Table 1. An effective antifungal of crude extracted from isopropanol at 713±0.7 mg/mL gave the most IZD against *C. gloeosporioides* with the clear zone 5.25±0.35 mm, against *C. acutatum* with the clear zone 11.00±4.24 mm, against *F. moniliforme* with clear zone 9.00±4.24 mm and against *F. oxysporum* with clear zone at 6.50±0.71 mm (Table 1). Methanol and isopropanol combination (1:1) gradient gave a yield 1.737±0.402 g/100 g of fresh fruit long pepper. It has an effective concentration 579±1.3 mg/mL against *C. gloeosporioides* with the clear zone 2.63±0.75 mm, against *C. acutatum* with the clear zone 10.25±0.11 mm, against *F. moniliforme* with the clear zone 8.38±1.70 mm and against *F. oxysporum* with the clear zone at 2.75±5.50 mm (Table 1).

CONCLUSION

This study shows that crude extract from the fruit of *P. retrofractum* Vahl using isopropanol or the gradient of methanol and isopropanol combination has a potency to inhibit fungal diseases. *Piper retrofractum*. The study of bioactive metabolites extracted from *P. retrofractum* shows that they are effective against plant disease-causing fungi *Fusarium moniliforme* DOAC 1224 causes the stalk rod disease, *F. oxysporum* DOAC 2269 causes the yellow wilt disease, *Colletotrichum gloeosporioides* DOAC 2213 and *C. acutatum* DOAC 2285 both cause the most destructive fungal disease, anthracnose. Total of crude extracted found in isopropanol is 2.193±0.579 g per 100 g of fresh pepper. Agar disc diffusion assay performed crude extracted concentrations 713 mg/mL against *C. gloeosporioides* is clear zone 5.25±0.35 mm. Against *C. acutatum* is clear zone 11.00±4.24 mm, against *F. moniliforme* is clear zone 9.00±4.24 mm and against *F. oxysporum* is clear zone at 6.50±0.71 mm. The results can be concluded that isopropanol is an effective solvent for extracting bioactive compounds from fresh *P. retrofractum*. The crude

metabolites have shown potential for broad range antifungal activities in plant pathogens. The results show potential in using extracted *P. retrofractum* as fungicides in organic farming and others. Furthermore, from the experiment, the solvent gave a high yield. Long pepper *P. retrofractum* Vahl is nontoxic. It is edible and has long been used as a spice ingredient in Thai food and as a herb in many recipes of Thai traditional medicine. The result of this study indicates that bioactive metabolite from *P. retrofractum* Vahl shows great potential as fungicides for agriculture in the future.

ACKNOWLEDGEMENT

Researchers express their sincere appreciation to Suan Sunandha Rajabhat University for support of this research.

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