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Anti-*Phytopthora capsici* Activities and Potential Use as Antifungal in Agriculture of *Alpinia galanga* Swartz, *Curcuma longa* Linn, *Boesenbergia pandurata* Schut and *Chromolaena odorata*: Bioactivities Guided Isolation of Active Ingredients

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Abstract: Problem statement: Plant derived fungicides are now being subjects of many research groups. These secondary metabolites have enormous potential to inspire and influence modern agrochemical research. The study aimed to investigate the antifungal activity and their potential use as fungicides in the agriculture of crude extracts and purified compounds derived from plants used in traditional medicines. **Approach:** Four medicinal plants including *A. galanga*, *C. longa*, *B. pandurata* and *C. odorata* were selected and percolated with hexane, ethyl acetate, acetone or methanol. The extracts were purified and elucidated their chemical structures. Disc mycelial growth inhibition was applied in order to determine their anti *P. capsici* activity and the field study was performed to determine their potential use in controlling fungal infection in chili plants compared with commercial fungicides such as captan and bio-control *Trichoderma virens*. **Results:** All crude extract inhibited mycelial growth of the fungus performed with similar efficacy. ED_{90} was equal to 300 ppm. Among plants studied *B. pandurata* was the most potent against *P. capsici*. The proposed active ingredients were pinostrobin and pinocembrin. In the field study, pinocembrin mediated the same anti *P. capsici* activity as captan. *B. pandurata* can protect chili from infection, thus increasing crop yield of chili comparable to *Trichoderma virens*. **Conclusion:** The results clearly showed that the extracts of the four plants studied could be considered as potential sources of novel fungicides. Particularly, *B. pandurata* has a very high potential as raw material for developing the antifungal molecule of non-petrochemical, naturally eco-friendly, easily obtainable and not toxic to human beings and environment, at least for use in chili growing.

Key words: *Phytophthora capsici*, *Alpinia galanga* swartz, *Curcuma longa* linn, *Boesenbergia*, *pandurata* schut, *Chromolaena odorata*, fungicides

INTRODUCTION

P. capsici is a soilborne pathogen and a serious threat to production of susceptible crops worldwide including many types of squash, watermelon, cucumber, peppers, chili, eggplant[1-3]. It is a fast spreading, aggressive disease, capable of causing complete crop failures. However, epidemics of *P. capsici* is largely dependent upon climate and soil conditions[4]. Many strategies to control diseases have been investigated in the field such as soil amendments and composts, bio-controls and applications of chemical fungicides.

Chemical control strategies should be integrated with cultural practices to manage Phytophthora blight. Chemical control measures commonly involve soil drenches early in the season and foliar applications during the season. Azoxystrobin, dimethomorph, fosetyl-Al and fluazinam were demonstrated as

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potential chemical management tools for Phytophthora root and stem rot of chili pepper plants[5]. However, applications of the synthetic fungicides usually take long timelines to be degraded completely causing heavy toxicity to human being and domestic animals[6]. In fact, a variety of different chemical and synthetic compounds have been used as fungicides such as benzimidazoles[7], alkaloids[8], artemisinin[9] and essential oils[10]. Research into plant derived fungicides for their applications in agriculture is now being intensified as these are having enormous potential to inspire and influence modern agrochemical research. There is a good reason to suppose that the secondary metabolites of plants have evolved to protect them from attack by the phytopathogens[11]. Ancient traditional use of plants as medicines provide the basis for indicating phytochemicals and plant extracts may be useful for specific medical conditions. And plant extracts have also been used as topical antiseptics, or have been reported to have antimicrobial activity[12,13].

A. galanga, *C. longa*, *B. pandurata* and *C. odorata* have been used in Thai traditional medicine and the first three plants are used in Thai cuisine. Certainly, their pharmacological and toxicological studies have been proved. However, the anti *P. capsici* activity has not been evaluated before. It is important to investigate those plants which have been used in traditional medicines as potential sources of novel fungicides.

Therefore, the crude extracts obtained from different solvent extraction of the plants and fractionations of the most potent crude extracts of each plant were evaluated for their anti *P. capsici* activity of both using disc mycelial growth inhibition and for field study.

MATERIALS AND METHODS

Plant materials: *A. galanga* (BKF 102287), *C. longa* (BKF 128478), *B. pandurata* (BKF 68909), *C. odorat*a (BKF 109181) were collected in Lampang province, Thailand during June and October 2007. A voucher specimen is deposited at Herbarium SAAR, Saar-Bangkok (BKF no.). Plant materials were dried at room temperature.

Extraction and isolation: Most plants were percolated with kinds of solvents e.g. hexane, ethyl acetate, acetone and methanol. The solutions were filtered using Millipore paper filter no. 4 then evaporated and crude extracts were stored under $N_{2\ (g)}$ atmosphere at-20°C. Furthermore, the crude extracts were tested for biological activity with *P. capsici* by poison food technique method. The most highly efficient extract of each plant was first subjected to coarse separation by column chromatography and crystallization.

***A. galanga* and *C. longa*:** The crude extracts were purified in open column chromatography using silica gel of 800 g (No. 7734, Mesh 70-230 ASTM, Merck) as the stationary phase. Gradient elution was conducted initially with *n*-hexane, gradually enriched with ethyl acetate, followed by increasing amount of methanol in ethyl acetate and finally ethyl acetate. Fractions were collected (500 mL) and combined on the basis of their TLC behavior. The solutions were evaporated until to obtain a powder form.

***B. pandurata*:** The crude extract of *B. pandurata* obtained from ethyl acetate was purified in an open column chromatography using silica gel of 1,300 g (No. 7734, Mesh 70-230 ASTM, Merck) as stationary phase. Gradient elution was conducted initially with n-hexane, gradually enriched with ethyl acetate, followed by increasing amount of methanol in ethyl acetate and finally ethyl acetate. Fractions were collected (500 mL) and combined on the basis of their TLC behavior. The solutions were evaporated until to obtain a powder form.

***C. odorata*:** The crude extract (96 g) of *C. odorata* obtained from the acetone phase was purified in open column chromatography using silica gel of 450 g (No. 7734, Mesh 70-230 ASTM, Merck) as stationary phase. Gredient elution was conducted initially with n-hexane, gradually enriched with ethyl acetate, followed by an increasing amount of methanol in ethyl acetate and finally ethyl acetate. Fractions were collected (500 mL) and combined on the basis of their TLC behavior. The solutions were evaporated until to obtain a powder form.

Spectroscopic characterization:
5-Hydroxy-7-methoxyflavanone (Pinostrobin)[14]:
Crystals: Colorless needles from EtOH m.p. 100.8-101.6 °C (Lit. m.p. 100-101°C).

UV λ_{max}^{MeOH} **nm (log ε):** 288 (4.10), 323 (3.83).

FTIR υ_{max}^{KBr} **cm^{-1}:** 3449 (O-H stretching), 1647 (C = O stretching of ketone), 1612, 1580, 1529, 1490, 1303, 1159.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.03 (*s*, 1H, OH-5), 7.45 (1H, m, H-2′,6′), 7.42 (1H, m, H-3′, 5′), 7.40 (1H, m, H-4′), 6.08 (d, 1H, J = 2.3 Hz, H-8), 6.06 (d, 1H,

J = 2.3 Hz, H-6), 5.43 (dd, 1H, J = 13.03, 3.01 Hz, H-2), 3.09 (dd, 1H, J = 17.18, 13.05 Hz, H-3a), 2.82 (dd, 1H, J = 17.17, 3.04 Hz, H-3b).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 195.80 (C-4), 168.01 (C-7), 164.17 (C-5), 162.81 (C-9), 138.38 (C-1′), 128.91(C-3′-5′), 126.16 (C-2′, 6′), 101.71(C-10), 95.85 (C-6), 78.29 (C-2).

EIMS m/z (% relative intensity): 270 (M^+, 76.81), 250 (14.31), 193 (100), 166 (26.61), 138.41 (29), 167 (12.99), 166(26.56), 138(31.09), 110(11.32), 95(20.37), 77(6.43), 50(5.12).

5-Hydroxy-7-hydroxyflavanone (Pinocembrin)[14]:
Crystals: yellow needles from EtOH m.p. 201.7-202.2°C (Lit. m.p. 200-201°C).

UV λ_{max}^{MeOH} **nm(log ε):** 288 (4.10), 323 (3.83).

FTIR υ_{max}^{KBr} **cm^{-1}:** 3415 (O-H stretching), 1630 (C = O stretching of ketone), 1583, 1487, 1467, 1357, 1302, 1089.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.13 (*s*, 1H, 5-OH), 10.85 (br *s*, 1H, 7-OH), 7.41 (1H, m, 2′, 3′, 4′, 5′, 6′), 5.93 (d, 1H, J = 2.1 Hz, H-8), 5.90 (d, 1H, J = 2.1 Hz, H-6) 5.58 (dd, 1H, J = 13, 3 Hz, H-2), 3.23(dd, 1H, J = 18, 13 Hz, H-3a), 2.72(dd, 1H, J = 18, 3 Hz, H-3a).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 195.81 (C-4), 166.63 (C-7), 163.42 (C-5), 162.63 (C-9), 138.61 (C-1′), 128.44(C-3′-5′), 126.49 (C-2′, 6′), 101.71 (C-10), 95.85 (C-6), 78.29 (C-2), 42.07(C-3).

EIMS m/z (% relative intensity): 256 (M^+, 64.73), 255 (100), 238 (29.91), 213 (4.60), 179 (73.29), 152 (16.92), 124 (25.01), 103(8.14), 96 (11.27), 78(13.08), 69(11.87), 50(8.83).

***trans, trans*-Curcumin:**
Crystals: Yellow needles from EtOH m.p. 185.1-186.2°C.

UV λ_{max}^{MeOH} **nm(log ε):** 364 (3.76).

FTIR υ_{max}^{KBr} **cm^{-1}:** 3504 (O-H stretching), 1628 (C = O stretching of conjugated ketone), 1601, 1510, 1459, 1428, 1280-1159 (C-O stretching), 1029, 961.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.59 (d, 1H, J = 15.75 Hz, H-2), 7.12 (dd, 1H, J = 8.17, 1.82 Hz, H-6′), 7.05 (d, 1H, J = 1.75 Hz, H-2′), 6.93 (d , 1H, J = 8.17 Hz, H-5′), 6.48 (d , 1H, J = 17.75 Hz, H-1), 5.90 (br s, 1H, H-4′), 5.80(s, 1H, H-4), 3.95(s, 3H, OCH_3-3′).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 183.28 (C-3, 5), 147.85 (C-4′), 146.79 (C-3′), 140.56 (C-2), 127.70 (C-1′), 122.89(C-6′), 121.78 (C-1), 114.84 (C-5′), 101.20 (C-4), 109.63 (C-2′), 55.97 (OCH_3-3′).

EIMS m/z (% relative intensity): 368 (21.35), 350(87.12), 191(100), 177(60.59).

4-Hydroxy-6, 7, 8, 4′-tetramethoxychalcone:
Crystals: yellow needles from EtOH m.p. 142.3-143.7°C.

UV λ_{max}^{MeOH} **nm(log ε):** 320 (4.43), 228 (4.45), 208 (4.52).

FTIR υ_{max}^{KBr} **cm^{-1}:** 3351, 3168 (O-H stretching), 1629 (C = O stretching of conjugated ketone), 1600, 1548, 1508, 1447, 1245-1151 (C-O stretching), 1019, 861.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 13.76 (*s*, 1H, 4-OH) 7.84 (*s*, 1H, H-1, 2), 7.61 ($AA'BB'$, 1H, J = 8.7, 2.5 Hz, H-2′, 6′), 6.95 ($AA'BB'$, 1H, J = 8.7, 2.5 Hz, H-3′, 5′), 6.30 (*s*, 1H, H-5), 3.93(*s*, 3H, OCH_3-6), 3.91 (*s*, 3H, OCH_3-8), 3.89 (*s*, 3H, OCH_3-4′), 3.83 (*s*, 3H, OCH_3-7).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 192.86 (C-3), 162.64 (C-4), 161.56 (C-4′), 159.95 (C-8), 155.0 (C-6), 143.41 (C-1), 135.31(C-7), 130.5 (C-2′, 6′), 128.05 (C-1′), 124.05 (C-2), 114.45 (C-3′,5′), 108.79 (C-9), 96.61 (C-5), 61.93 (OCH_3-6), 61.32 (OCH_3-7), 56.10 (OCH_3-8), 55.43 (OCH_3-4′).

ESI-MS: m/z 367.15 $[M+Na]^+$ (100%), 367.16 (28.08%), 345.1694 (5.16%), 280.97 (4.45%), 132.97 (15.49%), 118 (10.33%).

***P. Capsici* inoculum preparation:** The *P. capsici* isolated from diseased chili plants in Nan Province, Thailand was used as the pathogen of this study. The inoculums were prepared by transferring the isolate from V-8 juice broth to a jar containing vermiculite amended with 350 mL of V8 broth and incubated at 24°C for 4 weeks. The vermiculite inoculum containing sporangia and mycelia was placed on a sterile Petri-disc. The mycelia was cut into small pieces using a sterile blade and then transferred to a sterile molten medium and further incubated at 25°C for 10 days. The

isolate was maintained on Potato Dextrose Agar (PDA) (potato infusion from 200-20 g L^{-1} dextrose and 15 g L^{-1} agar) in the dark at 25°C.

Effect of compounds on mycelial radial growth: Mycelial discs (6 mm in diameter) of test fungi grown on PDA plates were cut from the margins of the colony and placed on PDA plates containing different concentrations of compounds (0, 1,000, 2,000, 3,000 and 4,000 ppm) and 3,000 ppm captan. After incubation at 25°C for 3-10 days, mycelial radial growth was measured and activity was expressed as EC_{50} (the concentration required for growth inhibition by 50%) as exclusively described by[15,16]. All experiments were repeated two times with three replicates. The results are presented as means ± SD. Multiple statistical comparisons were performed using SPSS program version 11.5 for Window.

Plants and treatments: Seedlings were fertilized with 20-10-20 of N-P_2O_5-K_2O (Scotts-Sierra Horticultural Products Co., Marysville) nutrient solution through a Dosatron® (Dosatron Intern., Clearwater, FL) dispenser at 1:100 dilution until transplanted. Four-week old seedlings were transplanted to 15 cm-diameter clay pots (1700 mL in volume) and placed on saucers on greenhouse benches for 2-4 weeks before used.

Plants were grown in non-infested and pathogen-infested soil and covered with clay plastic mulch. Raised beds (20 cm high × 20 cm wide), spaced 50 cm apart (center to center) were manually constructed, amended with composts and soil amendments, followed by the application of napropamide (Riedel de Haën; Seelze, Germany). The fractions and pure compounds that were found very active against *P. capsici* in culture system were studied in the field. Plants were grown in non-infested and pathogen-infested soil and covered with clay plastic mulch. Raised beds were divided into 8 groups including:

T1 = Treatment using 20 g L^{-1} acetone fraction of *C. odorata* in 50% ethanol 60 mL
T2 = Treatment using 20 g L^{-1} hexane fraction of *A. galanga* in 50% ethanol 60 mL
T3 = Treatment using 1 g L^{-1} trans, trans-curcumin in 50% ethanol 20 mL
T4 = Treatment using 20 g L^{-1} ethyl acetate fraction of *B. pandurata* in 50% ethanol 60 mL
T5 = Treatment using 1 g L^{-1} pinocembrin in 50% ethanol 20 mL
T6 = Treatment using 1 g L^{-1} captan in distilled water 30 mL
T7 = Treatment using 2 g *Trichoderma virens* infested in soil before plant growing
T8 = treatment using distilled water

Plants were watered with solutions of the compounds only one time immediately after planting with. The efficacy of the compound was measured by counting the surviving plants at 1, 3, 5 and 7 weeks after treatments. In each test, a completely randomized block design was used. One crop of fresh chili fruits were harvested and weighed for each treatment. The yield of each treatment was compared by using Duncan's Multiple Range Test ($p<0.05$). Three replicates served for soil sampling for microbial activity and ten for disease evaluation.

RESULTS

Isolation of crude extracts and anti *P. capsici* activity: Dried plants of 5 kg were ground and percolated with 18 liters of given solvents (3 rounds of 3-day interval at room temperature). The extractions were begun using the solvents by increasing their polarity such as hexane, ethyl acetate, acetone and methanol, respectively. The yield of the extraction process was monitored by weighing the extracts in powder form after evaporation. The chemical composition of plants found in each solvent varied from plant to plant. The crude contents obtained from hexane of all plants were obviously less than those found in ethyl acetate fractions except for *C. odorata* (Table 1).

The antifungal activity of crude extracts obtained from hexane, ethyl acetate, acetone and methanol fraction varied in greatly. Among fractions tested, the highest activity was the hexane fraction for *A. galanga* (86% inhibition for 1,000 ppm) the acetone fraction for *C. longa* (62% inhibition for 1,000 ppm), the ethyl acetate fraction for *B. pandurata* (74% inhibition for 1,000 ppm) and acetone fraction for *C. odorata* (100% inhibition for 2,000 ppm).

Table 1: Extraction yield of plants. Dried plants (5,000 g) were percolated with hexane, ethyl acetate, acetone or methanol

		Weight of crude extract (g)			
Plants	Dried weight (g)	Hexane	Ethyl acetate	Acetone	Methanol
A. galanga	5,000	72.84	89.35	52.92	115.38
C. longa	5,000	202.49	479.68	165.32	82.25
B. pandurata	5,000	101.84	279.98	54.87	77.89
C. odorata	5,000	148.47	307.96	561.92	288.66

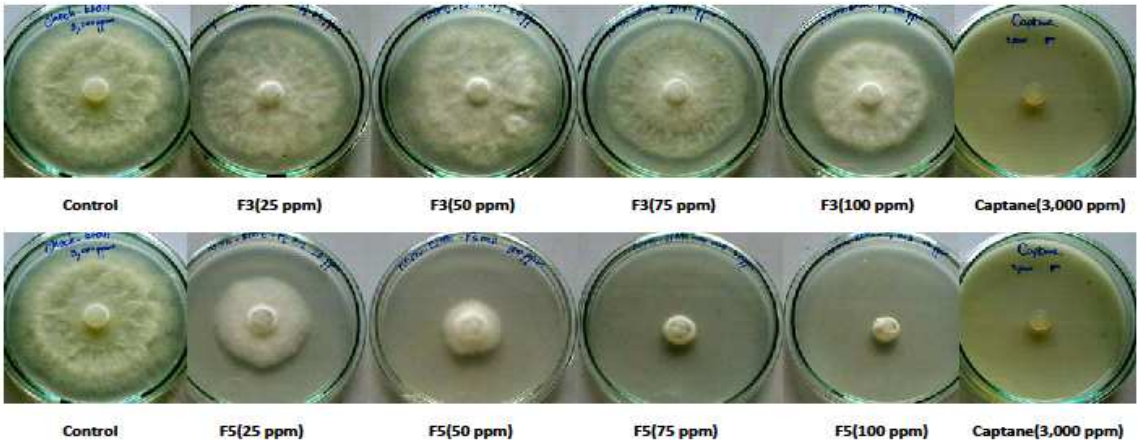

Fig. 1: Inhibition of mycelial growth activity of (a): F3 and (b): F5 compounds from the crude extract of *B. pandurata*

Table 2: Inhibition of mycelial radial growth (%) of F1, F2 and F3 separated from the hexane fraction of *A. galanga*

	Percentage of Inhibition of *P. capsici* radial growth					
	F_1		F_2		F_3	
Treatment	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
*Control	0.00	0.00	0.00	0.00	0.00	0.00
**Check	5.21	3.55	5.21	3.55	5.21	3.55
***Captan	100.00	0.00	100.00	0.00	100.00	0.00
100 ppm	23.45	8.17	34.34	5.02	33.18	3.46
200 ppm	56.02	7.95	64.32	12.22	49.36	8.13
300 ppm	64.57	21.74	100.00	0.00	100.00	0.00
400 ppm	100.00	0.00	100.00	0.00	100.00	0.00

*: Contains only PDA medium; **: Contains PDA medium and ethanol (3000 ppm); ***: Contains PDA medium, ethanol (3000 ppm) and Captan (100,000 ppm)

Purification of the active in-gradients: The most potent crude extracts of each plant were selected and submitted to purification by an open column chromatography using silica gel as stationary phase. The columns were eluted by using solution of ethyl acetate-hexane and methanol-ethyl acetate. The fractions were collected and evaporated and the inhibition of mycelial radial growth of *P. capsici* was investigated. Figure 1 demonstrates the typical results of inhibition of the mycelial radial growth of *P. capsici* of the F3 and F5 isolated from the hexane phase of *B. pandurata* compared with captan. Captan (1, 2, 3, 6-tetrahydro-N-(trichloromethylthio) phthalimide) is used to control fungal disease on a wide variety of crops and seeds. It also has a broader industrial application for control of mould in paints, lacquers and wallpaper pastes[17].

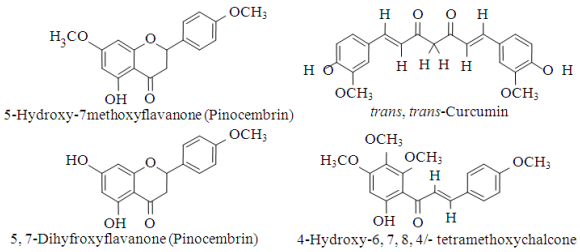

Fig. 2: Chemical structures of the proposed active ingredients

***A. galanga*:** The F1, F2 and F3 fraction isolated from crude extract of hexane phase revealed anti *P. capsici* to be about 300 fold more efficient than captan as indicated in Table 2.

***C. longa*:** The crude extract of *C. longa* obtained from acetone phase consisted of six fractions, F1, F2, F3, F4 and F5 fraction. All compounds revealed anti *P. capsici* to be about 300 fold more efficient than captan as indicated in Table 3.

***B. Pandurata*:** The crude extract of *B. pandurata* obtained from ethyl acetate phase was composed of nine fractions, F1, F2, F3, F4, F5, F6, F7, F8 and F9. All compounds revealed anti *P. capsici* to be about 300 fold more efficient than captan as indicated in Table 4.

***C. odorata*:** The crude extract of *C. odorata* obtained from acetone phase was composed of five fractions, F1, F2, F3, F4 and F5 were separated. All compounds revealed anti *P. capsici* to be about 300 fold more efficient than captan as indicated in Table 5.

Table 3: Inhibition of mycelial radial growth (%) of F_1, F_2, F_3, F_4, F_5 and F_6 separated from the acetone fraction of *C. longa*

	Percentage of Inhibition of *P. capsici* radial growth									
	F_1		F_2		F_3		F_4		F_5	
Treatment	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
*Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
**Check	50.89	54.63	50.89	54.63	50.89	54.63	50.89	54.63	58.67	52.88
***Captan	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
100 ppm	30.38	36.36	10.73	9.75	41.91	3.80	60.31	34.23	40.83	17.49
200 ppm	68.36	30.67	95.81	11.04	76.92	17.02	85.00	12.80	71.10	21.52
300 ppm	88.74	15.12	100.00	0.00	100.00	0.00	100.00	0.00	80.70	26.27
400 ppm	95.70	9.05	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00

*: Contains only PDA medium; **: Contains PDA medium and ethanol (3000 ppm); ***: Contains PDA medium, ethanol (3000 ppm) and Captan (100,000 ppm)

Table 4: Inhibition of mycelial radial growth (%) of F_1, F_2, F_3, F_4, F_5, F_6, F_7, F_8 and F_9 separated from the ethyl acetate fraction of *B. pandurata*

	Percentage of inhibition of *P. capsici* radial growth																	
	F_1		F_2		F_3		F_4		F_5		F_6		F_7		F_8		F_9	
Treatment	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Control*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Check*	10.26	5.05	10.26	5.05	10.26	5.05	10.26	5.05	10.26	5.05	10.26	5.05	57.64	13.94	57.64	13.94	2.42	3.36
Captane*	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
100 ppm	11.69	7.13	18.26	7.85	16.93	10.76	26.78	10.79	73.96	2.39	46.78	6.92	36.64	16.40	21.94	1.36	13.04	3.49
200 ppm	30.36	16.81	29.58	10.68	32.43	6.31	56.59	8.90	89.91	7.47	65.96	4.47	79.93	14.48	61.95	0.95	42.27	5.22
300 ppm	75.48	7.03	62.79	11.89	70.98	9.75	82.79	12.86	85.81	9.35	87.08	9.53	85.06	14.69	68.54	3.67	78.10	16.12
400 ppm	94.74	7.81	62.20	19.91	93.43	9.96	95.70	6.29	100.00	0.00	100.00	0.00	81.18	19.07	100.00	0.00	76.98	21.83

*: Contains only PDA medium; **: Contains PDA medium and ethanol (3000 ppm); ***: Contains PDA medium, ethanol (3000 ppm) and Captan (100,000 ppm)

Table 5: Inhibition of mycelial radial growth (%) of F_1, F_2, F_3, F_4 and F_5 separated from the acetone fraction of *C. odorata*

	Percentage of inhibition of *P. capsici* radial growth									
	F_1		F_2		F_3		F_4		F_5	
Treatment	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
*Control	0.000	0.00	0.000	0.000	0.00	0.000	0.00	0.00	0.000	0.00
**Check	3.066	3.84	3.066	3.848	3.06	3.848	3.06	3.84	3.066	3.48
***Captan	100.000	0.00	100.000	0.000	100.00	0.000	100.00	0.00	100.000	0.00
100 ppm	26.110	7.89	14.910	11.610	36.25	7.240	60.66	6.62	7.890	2.03
200 ppm	65.500	6.38	55.070	3.925	56.48	8.880	98.50	0.00	9.680	2.50
300 ppm	100.000	0.00	100.000	0.000	100.00	0.000	100.00	0.00	92.850	10.68
400 ppm	100.000	0.00	100.000	0.000	100.00	0.000	100.00	0.00	100.000	40.75

*: Contains only PDA medium; **: Contains PDA medium and ethanol (3000 ppm); ***: Contains PDA medium, ethanol (3000 ppm) and Captan (100,000 ppm)

All compounds isolated from these active fractions were very potent antifungal agents particularly against *P. capsici* comparable with captan. The concentrations required to inhibit the mycelial radial growth of *P. capsici* by 90% (ED_{90}) were about 300 ppm for these compounds. It should be noted that the quantities of some compounds obtained were very small amounts, not enough for spectroscopic studies. Only four compounds that were abundantly obtained from rechromatographed and re-crystallized were selected for elucidating their chemical structures. The spectroscopic study suggested the chemical structure of compound F4 obtained from acetone fraction of *C. longa* was curcumin; compound F3 and F5 of ethyl acetate of *B. pandurata* was pinostrobin and pinocembrin, respectively and compound F4 of acetone fraction of *C. odorata* was 4-Hydroxy-6,7,8,4$^{/}$-tetramethoxychalcone (Fig. 2).

Field test: Figure 3 represents typical results of the series of experiments demonstrating the severity of *P. capsici* infected chili after transplanting in infested-pathogen soil. Two sets of control treatments were established; chili plants grown either in field soil

(a) (b) (c)

Fig. 3: Effects of sporangia and mycelia of *P. capsici* on chili plants: (a) dead and (b) survival chili plants at 3 weeks after transplanting in infested-pathogen soil without antifungal and (c) survival of chili after transplanting in infested-pathogen soil with single treatment using 2 g *Trichoderma virens*

infested with sporangia and mycelia of *P. capsici* with and without 50% ethanol of 60 mL after being transplanted into the soil. The first symptom on chili plants in the field is commonly crown rot. A lesion girdling the base of the stem causes rapid collapse and death of the plant. This phase of the disease usually occurs in low-lying areas of the field. Following rainstorms, black, girdling lesions form on the stem and in the extremities of chili branches, resulting in wilting of leaves and branches.

The affected plants gradually die. *P. capsici* caused severe damage to root and crown rot disease throughout all the series of experiments. Figure 3a represented typical results of the series of experiments demonstrating the severity of *P. capsici* infected chili after transplanting in infested-pathogen soil. Plants were infected and dead at 3 weeks after planting. The infected survival chilies were characterized by wilted plants, yellow leaves and minimal or absent crop yield (Fig. 3b). However, the treatments using antifungal compounds as well as the known antifungal including captan and bio-control *Trichoderma virens*[18-20] can significantly protect plants from infection (Fig. 3c). It should be noted that 20 g L^{-1} extract of hexane fraction of *A. galanga* and 1 g L^{-1} pinocembrin significantly increased the percentage of survival chili comparable with 1 g L^{-1} captan and 2 g *Trichoderma virens* (Table 6). Fresh chili fruits of each series of experiments were harvested and weighed as indicated in Table 7. As expected, for one crops harvest, the untreated series gave the lowest and the *Trichoderma virens* treated series gave the highest product yield. All fractions tested and captan increased crop yield of chili. Among the molecules used, pinocembrin was the most efficient for increasing crop yield.

Table 6: Surviving plants (%) after planting in *P. capsici*-infested soil with and without treatments

	Survival Plants (%) after treatment			
Treatments	1 week	3 week	5 week	7 week
T1	75.28	51.39bc[1]	38.89d[1]	33.33bc[1]
T2	91.94	59.72b	55.56ab	43.61abc
T3	85.28	58.33bc	44.44cd	34.72bc
T4	84.44	55.56bc	45.83cd	38.06bc
T5	88.33	59.72b	51.39bc	48.61ab
T6	92.50	58.34bc	53.61bc	47.22ab
T7	93.06	80.56a	63.89a	56.94a
T8	93.06[ns]	48.62c*	37.50d*	29.16c*
CV (%)	10.37	11.01	12.46	23.19

1: Mean value of the same column following the same letter is not statistically different when compared using Duncan's Multiple Range Test ($p \leq 0.05$); ns = Non statistically difference; *: Statistically difference at $p \leq 0.05$

Table 7: Crop production of fresh chili fruits (g) per raised bed

Treatments	Weight of fresh chili fruit of one crops harvest (g/raised bed)
T1	1,014.50d[1]
T2	1,407.25c
T3	999.00d
T4	1,505.00c
T5	2,674.75a
T6	2,365.00b
T7	2,959.00a
T8	923.00d

1: Mean value of the same column following the same letter is not statistically different when compared using Duncan's Multiple Range Test ($p<0.05$); CV: 10.15%

DISCUSSION

The study showed that *P. capsici* significantly caused an incremental death or a big loss of crop yield after transplanting chili plants in pathogen-infested soil without treatment compared with the treated series using extracts, captan or *Trichoderma virens*. The study also clearly showed that the extracts from *A. galanga*, *C. longa*, *B. pandurata* and *C. odorata* have potent anti *P. capsici* activity at *in vitro* mycelial growth inhibition and field study. It should be noted that the results of the field study were obtained by using only a single treatment at the beginning of transplanting chili to the pathogen-infested soil. Unfortunately, the chemical structure of the expected active ingredient of the crude extracts cannot be elucidated for *A. galanga*. Because the herb consists of a very small amount of active ingredient molecules, no further characterization and chemical structure can be elucidated. For *C. longa* and *C. odorata* the amounts of active ingredients were also small but sufficient only for spectroscopic study. The proposed active ingredient was curcumin and 4-Hydroxy-6, 7, 8, 4′-tetramethoxychalcone for *C. longa*

and *C. odorata*, respectively. Indeed, *B. pandurata* should be considered as a potential source of fungicide since the active ingredient compounds are abundantly found in pinostobin and pinocembrin.

The results clearly showed that pinocembrin mediated anti *P. capsici* activity similar to that of captan and slightly lower than *Trichoderma virens*. The results also suggested that the bio-control *Trichoderma virens* should be considered as a potential bio-control agent against *P. capsici*, a soilborne plant-pathogenic fungus. It was reported that captan acts through inhibition of the fungal process of respiration and metabolism through a non-specific thiol reactant. Although it is not a systemic fungicide, adjuvants can enhance transport of captan through a plant cuticle[21]. Captan is detected in fruit and vegetables during market basket monitoring and total diet studies[22]. These signify that captan can cause a serious toxicity for human beings and domestic animals.

CONCLUSION

The results clearly showed good correlation of the anti-fungal activity of extracts of the four Thai medicinal plants including *A. galanga*, *C. longa*, *B. pandurata* and *C. odorata* at disc mycelial growth inhibition and controlling fungal infection in chili plant studies. The extracts of these four plants could be considered as potential sources of novel fungicides. Since *B. pandurata* consists of high amounts of pinostrobin and pinocembrin, the proposed active ingredients. Thus, *B. pandurata* has a very high potential as raw material for developing the antifungal molecule of non-petrochemical, naturally eco-friendly, easily obtainable and not toxic to human beings and environment, at least for use in chili growing.

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