Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 3 (4): 237-245, 2008
ISSN 1816-4935

Synergistic Activities of 4-Arylcoumarins Against Phytopathogenic Fungi

[1]Thongchai Taechowisan, [2]Asawin Wanbanjob, [2]Pittaya Tuntiwachwuttikul,
[3]Yuemao Shen and [4]Saisamorn Lumyong
[1]Department of Microbiology,
[2]Department of Chemistry, Faculty of Science, Silpakorn University,
Nakorn Pathom 73000, Thailand
[3]Kunming Institute of Botany, The Chinese Academy of Sciences,
Kunming 650204, China
[4]Department of Biology, Faculty of Science, Chiang Mai University,
Chiang Mai 50200, Thailand

Abstract: Different extracts of *Streptomyces aureofaciens* CMUAc130 culture were studied as potential antifungal agents for selected phytopathogenic fungi. In a serial agar dilution method, crude ethyl acetate and 10% methanol in ethyl acetate extracts exhibited fungistatic activity against *Aspergillus flavus*, *Colletotrichum musae*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Both ethyl acetate extract and 10% methanol in ethyl acetate extract were highly effective on all tested fungi, with Minimum Inhibitory Concentration (MIC) values ranging from 0.25 to 50 and 10 to 100 mg mL^{-1}, respectively. The major active ingredients from those extracts were purified by silica gel column chromatography and identified to be 5, 7, 4'-trimethoxy-4-phenylcoumarin (1), 4'-hydroxy-5,7-dimethoxy-4-phenylcoumarin (2), 3'-Hydroxy-5,7,4'-trimethoxy-4-phenylcoumarin (3), 5,7,3',4'-Tetramethoxy-4-phenylcoumarin (4) and 4'-hydroxy-5,7,3'-trimethoxy-4-phenylcoumarin (5) by NMR and mass spectral data, respectively. Five compounds (1 to 5) had activity against *F. oxysporum* with MICs of 0.30, 1.00, 0.40, 10.00 and 20.00 mg mL^{-1}, respectively. Compounds 1, 2 and 3 also showed a synergistic effect when combined in different concentrations, displaying four times less concentration to reach complete inhibition in the growth of *F. oxysporum*.

Key words: 4-arylcoumarins, antifungal activity, phytopathogenic fungi, synergistic activity

INTRODUCTION

Plant pathogens, particularly fungi, are responsible for yield reductions in food and crops throughout the world. Although these losses may be attenuated by the use of resistant cultivars, crop rotation, or sanitation practices, fungicides are often also needed to maximize crop yields (Knight *et al*., 1997). These antifungal chemicals also contribute substantially to the quality of food and human health by controlling many of the fungi that produce mycotoxins (Dmello *et al*., 1999) or by interfering in their biosynthesis (Hasan, 1999). Despite these significant benefits, synthetic fungicides are also responsible for the generation of toxic residues (Hall, 1984) and the development of resistance in pathogens (Wilson *et al*., 1997). These is therefore a continuing need to develop and release new fungitoxic chemicals that preserve the environment and enable a more efficient control of pathogenic fungi, improving crop yield and quality.

Corresponding Author: Thongchai Taechowisan, Department of Microbiology, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand
Tel: +66 34 243429/8809 Fax: +66 34 273046

In earlier study the organism *Streptomyces aureofaciens* CMUAc130 was isolated from root tissue of *Zingiber officinale* Rosc. (Zingiberaceae). Cultures produced 5, 7, 4'-Trimethoxy-4-phenylcoumarin 1 and 4'-Hydroxy-5,7-dimethoxy-4-phenylcoumarin 2 which had antifungal activities (Taechowisan *et al*., 2005). In the present paper, the other 4-arylcoumarins were isolated, 3'-hydroxy-5,7,4'-trimethoxy-4-phenylcoumarin 3, 5,7,3',4'-tetramethoxy-4-phenylcoumarin 4 and 4'-hydroxy-5,7,3'-trimethoxy-4-phenylcoumarin 5, we report the fungistatic and fungicidal activity exhibited by 4-arylcoumarins on several phytopathogenic fungi, among them, *Aspergillus flavus*, *Colletotrichum musae*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. These fungi were selected because of their importance in causing major plant diseases affecting crop yields and quality. The inhibitory activity of each isolated compound and their combinations on the fungal growth are also described.

MATERIALS AND METHODS

Organisms and Media

S. aureofaciens CMUAc130 was isolated from root tissues of *Z. officinale* by the surface-sterilization technique (Taechowisan *et al*., 2003). Identification of the isolate to species level was based on morphological, cultural, physiological and biochemical characteristics and also 16S rDNA gene sequencing as described by Taechowisan and Lumyong (2003). Spores of *S. aureofaciens* CMUAc130 were used to inoculate 200 plates of International *Streptomyces* Project (ISP) Medium 2 and incubated for 14 days at 28°C (Shirling and Gottlieb, 1966). The culture medium was then cut into small pieces that were extracted with organic solvent (3×500 mL); hexane, ethyl acetate and 10% methanol in ethyl acetate, respectively. Each of organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown oil (hexane extraction, 658.6 mg) and a dark brown solid (ethyl acetate extraction, 852.4 mg and 10% methanol in ethyl acetate extraction, 583.5 mg). Isolates of *A. flavus* obtained from peanut, *C. musae* from banana, *P. ultimum* from potato, *R. solani* from tomato, *F. oxysporum* from wheat and *S. rolfsii* from maize were used. All fungi were cultured on Potato Dextrose Agar (PDA) medium.

Fractionation and Purification of the Compounds

The mixture residue of 350 mg of ethyl acetate and 10% methanol in ethyl acetate extraction was dissolved in 10 mL methanol and fractionated on a reverse phase column (Li Chroprep RP-18, Merck, Germany) with increase the concentration of methanol as eluent (50, 70 and 100%). The fractions were combined on the basis of TLC results and concentrated under the vacuum to produce the black gum (180 mg). The black gum was mixed with 2.0 g of silica gel (Walk gel). This mixture was then subjected to bioassay-guided separation through column (50×7.5 cm) chromatography over silica gel (50 g) eluted with chloroform-methanol (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 0:100). About 50 mL of each fraction was collected. Purifications were performed by column chromatography over silica gel (finer than 200 mesh). The fractions were combined and made into four pooled fractions on the basis of their TLC results. The fractions, A-1 (eluted with chloroform and methanol 95:5 to 90:10), A-2 (eluted with chloroform and methanol 90:10 to 80:20), A-3 (eluted with chloroform and methanol 80:20 to 60:40) and A-4 (eluted with chloroform and methanol 60:40 to 30:70) were screened again for their antifungal activity (Taechowisan *et al*., 2005). The fractions A-2 and A-3 were most active against those fungi and they showed one major spot having the same Rf value (0.63, chloroform and methanol 20:1) in TLC. Since they were identical, we combined these fractions and by purified them by repeated chromatography over a silica gel column, followed by recrystallization in a mixture of hexane and chloroform (9:1). This yielded the active compounds 1 and 2. Purification of A-1 resulted respectively in compounds 3 and 4. Purification of A-4 resulted in compound 5. The structures of the active compounds have been identified using NMR and mass spectral data.

Structure Elucidation of the Compounds

The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^{1}H and ^{13}C NMR spectra on a Bruker DRX 500 spectrometer and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Data Analysis

Data of antifungal activity assay and minimum inhibitory concentration of CMUAc130, were analyzed by SPSS for window 11.01 (SPSS Inc., Chicago, USA). Means of treatments for each experiment were compared using Duncan's multiple range test ($p \leq 0.05$).

Antifungal Assays

The crude extracts and the purified compounds were evaluated for their antifungal activity, using an agar serial dilution method according to Shadomy *et al.* (1985). Inocula were prepared by suspending conidia from 48-96 h old cultures (1×10^{6} conidia mL^{-1}) in physiological saline solution. Similar size (1-2 mm) mature sclerotia of *S. rolfsii* weighing 5.80-14.3$\times10^{-4}$ g were also used for the assays.

Bioassay were conducted by adding the appropriate amounts of the crude extract or the purified compounds, to 5 mL of 10% glucose medium, to obtain a final concentration of 0.25-200 mg mL^{-1}. The fungicide MCZ (ethylenebis(dithiocarbamic acid) manganese zinc complex) was used for the antifungal assays. Dilutions of the crude extract were prepared by previously dissolving in methanol (final concentration of methanol < 10%), while the purified compounds were dissolved in chloroform (final concentration of $CHCl_3$ < 4%) before mixing with the glucose media. Plates containing culture medium, with or without addition of the above-mentioned solvents, were also used as controls for each fungus.

A broth micro dilution method (Wilson *et al*., 1997) was used to determine the MIC of the purified compounds individually or in different combinations. Four day old conidia from a monosporic culture of *F. oxysporum* were added to glucose-mineral salts medium to reach 1×10^{5} conidia mL^{-1}. Purified compounds dissolved in dimethyl sulfoxide (DMSO) were incorporated into each well, by duplicate, containing the spore suspension, resulting in concentrations ranging from 0.025 to 1500 mg mL^{-1}. The final concentration of DMSO did not exceed 2-4%. Wells containing spore suspension with or without addition of DMSO were simultaneously made as controls. The percentage of fungi growth (recorded in percentage from 0 to 100) was visually determined with an inverted light microscope for each concentration of the compounds. The measurements were always done by the same operator. The inhibitory concentration (IC_{50}) values were calculated by Probit analysis on the basis of percentage of inhibition obtained at each concentration of the samples.

Synergism among the pure compounds was also measured, adding to each well different combinations of different concentrations of each compound to be tested. In all cases, the final concentration of DMSO was lower than 8%.

Minimum Fungicide Concentration (MFC). These tests were carried out to analyze the possible fungicidal activity exhibited by the crude extract and the purified compounds, as well as studying their fungistatic activity. Fourteen days after the beginning of the MIC assay, a circle of agar around the culture center as well as sclerotia of *S. rolfsii*, from those plates that exhibited negative growth were obtained. Two replicates of the agar circle or the sclerotia were placed in the center of Petri dishes containing adequate medium for each fungi and then incubated for 14 days. At the end of this period, the MFC values were recorded.

RESULTS AND DISCUSSION

Isolation of the Antifungal Active Compounds

The crude extract by ethyl acetate (350 mg) of *S. aureofaciens* CMUAc130 was subjected to silica gel column chromatography and four pooled fractions, A-1, A-2, A-3 and A-4, were obtained. All the fractions performed their antifungal activity. These active fractions were then subjected to repeated chromatography, followed by recrystallization in a mixture of hexane and chloroform (9:1) and then afforded compound 1 (27 mg) and 2 (20 mg) in the mixture of fractions A-2 and A-3. Purification of A-1 resulted in compound 3 (21 mg) and compound 4 (9 mg). Purification of A-4 resulted in compound 5 (7 mg) (Fig. 1).

Structure Elucidation of Compound 1

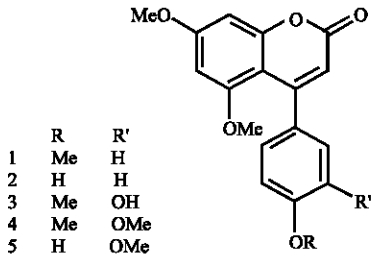
5, 7, 4'-Trimethoxy-4-phenylcoumarin ($C_{18}H_{16}O_5$) was a white amorphous powder: mp 151-152°C (EtOH); UV λ_{max} (MeOH) nm (log ϵ): 250 (4.07), 325 (4.29); ^{1}H NMR ($CDCl_3$):δ7.20 (2H, d, J = 8.5 Hz, H-2', H-6'), 6.87 (2H, d, J = 8.5 Hz, H-3', H-5'), 6.50 (1H, d, J = 2.5 Hz, H-8), 6.22 (1H, d, J = 2.5 Hz, H-6), 5.96 (1H, s, H-3), 3.83 (6H, s, OMe-7, OMe-4'), 3.46 (3H, s, OMe-5); IR ν_{max} ($CHCl_3$) cm^{-1}: 1710, 1610, 1595, 1510, 1158, 1111, 1052, 952, 872, 860, 830; MS m/z (rel. int.): 312 $[M]^+$ (80), 284 $[M - CO]^+$ (100), 269 $[M - MeCO]^+$ (37), 241 $[M - 43-CO]^+$ (2).

Structure Elucidation of Compound 2

4'-Hydroxy-5,7-dimethoxy-4-phenylcoumarin ($C_{17}H_{14}O_5$) was a white amorphous powder: mp 214-215°C (MeOH); UV λ_{max} (MeOH) nm (log ϵ):256 (4.04), 324 (4.22); UV λ_{max} (NaOMe) nm: 256, 368; ^{1}H NMR (CD_3COCD_3):δ8.50 (1H, s, exchangeable D_2O, OH-4'), 7.14 (2H, d, J = 8.5 Hz, H-2', H-6'), 6.82 (2H, d, J = 8.5 Hz, H-3', H-5'), 6.51 (1H, d, J = 2.5 Hz, H-8), 6.37 (1H, d, J = 2.5 Hz, H-6), 5.80 (1H, s, H-3), 3.91 (3H, s, OMe-7), 3.53 (3H, s, OMe-5); $\Delta\delta = \delta C_5D_5N-\delta CD_3COCD_3$ = H-2' + H-6' (+0.19), H-3' + H-5' (+0.32), H-8 (+0.11), H-6 (+0.01), H-3 (+0.38) OMe-7 (-0.16), OMe-5 (-0.23); IR ν_{max} ($CHCl_3$) cm^{-1}: 1708, 1612, 1598, 1512, 1159, 1112, 1054, 952, 870, 860, 832; MS m/z (rel. int.): 298 $[M]^+$ (100), 270 $[M-CO]^+$ (82), 255 $[M-MeCO]^+$ (29), 227 $[M-43-CO]^+$ (15).

Structure Elucidation of Compound 3

3'-Hydroxy-5,7,4'-trimethoxy-4-phenylcoumarin ($C_{18}H_{16}O_6$) was a white amorphous powder: mp 153-154°C (EtOH); UV λ_{max} (MeOH) nm (log ϵ):252 (4.13), 329 (4.32); UV λ_{max} (NaOMe) nm: 250, 288 sh, 329, 400 sh; ^{1}H NMR ($CDCl_3$):δ5.92 (1H, s, H-3), 5.87 (1H, s, exchangeable D_2O,



	R	R'
1	Me	H
2	H	H
3	Me	OH
4	Me	OMe
5	H	OMe

Fig. 1: Chemical structures of 5, 7, 4'-trimethoxy-4-phenylcoumarin (1), 4'-Hydroxy-5,7-dimethoxy-4-phenylcoumarin (2), 3'-hydroxy-5,7,4'-trimethoxy-4-phenylcoumarin (3), 5,7,3',4'-tetramethoxy-4-phenylcoumarin (4) and 4'-hydroxy-5,7,3'-trimethoxy-4-phenylcoumarin (5)

OH-3'), 3.86 and 3.78 (3H and 3H, s and s, OMe-7, OMe-4'), 3.43 (3H, s, OMe-5); IR v_{max} ($CHCl_3$) cm^{-1}: 3525, 1710, 1615, 1597, 1510, 1158, 1111, 1052, 945, 909, 859, 828; MS m/z (rel. int.): 328 $[M]^+$ (100), 300 $[M-CO]^+$ (51), 285 $[M-MeCO]^+$ (31), 257 $[M-43-CO]^+$ (17).

Structure Elucidation of Compound 4

5,7,3',4'-Tetramethoxy-4-phenylcoumarin ($C_{19}H_{18}O_6$) was a white amorphous powder: mp 169-170°C (MeOH); UV λ_{max} (MeOH) nm (log ϵ):248 (4.13), 328 (4.26); ^1H NMR ($CDCl_3$):δ6.93-6.73 (3H, complex, H-2',H-5', H-6'), 6.51 (1H, d, J = 2.5 Hz, H-8), 6.25 (1H, d, J = 2.5 Hz, H-6), 6.00 (1H, s, H-3), 3.91 and 3.85 (3H and 6H, s and s, OMe-7, OMe-3', OMe-4') and 3.48 (3H, s, OMe-5); IR v_{max} ($CHCl_3$) cm^{-1}: 1708, 1610, 1595, 1510, 1158, 1111, 1052, 944, 902, 855, 826; MS m/z (rel. int.): 342 $[M]^+$ (100), 314 $[M-CO]^+$ (40), 299 $[M-MeCO]^+$ (9), 271 $[M-43-CO]^+$ (2).

Structure Elucidation of Compound 5

4'-hydroxy-5,7,3'-trimethoxy-4-phenylcoumarin ($C_{18}H_{16}O_6$) was a white amorphous powder: mp 173-174°C (Et_2O); UV λ_{max} (MeOH) nm (log ϵ):251 (4.07), 329 (4.19); UV λ_{max} (NaOMe) nm: 250, 331, 395; ^1H NMR ($CDCl_3$):δ6.52 (1H, d, J = 2.5 Hz, H-8), 6.26 (1H, d, J = 2.5 Hz, H-6), 6.02 (1H, s, H-3), 3.88 and 3.86 (3H and 3H, s and s, OMe-7, OMe-7'), 3.36 (3H, s, OMe-5); IR v_{max} ($CHCl_3$) cm^{-1}: 3524, 1709, 1611, 1595, 1510, 1158, 1111, 1052, 943, 903, 855, 825; MS m/z (rel. int.): 328 $[M]^+$ (100), 300 $[M-CO]^+$ (66), 285 $[M-MeCO]^+$ (9), 257 $[M-43-CO]^+$ (3).

MIC of Extracts

The crude extracts of *S. aureofaciens* CMUAc130 culture were assayed for their potential antifungal activity on different phytopathogenic fungi. All extracts, depending on the concentration, exhibited inhibition on the growth of all of the tested i.e., fungi *A. flavus*, *C. musae*, *F. oxysporum*, *P. ultimum*, *R. solani* and *S. rolfsii*. The ethyl acetate extract exhibited a significant inhibition on fungal growth with a MIC ranging from 0.25 to 25 mg mL^{-1} while the 10% methanol in ethyl acetate extract showed a mild activity (MIC = 25-100 mg mL^{-1}) (Table 1). Hexane extract showed a low antifungal activity with MIC values from 50-200 mg mL^{-1}, which means that low hydrophobic antifungal substances (oils) had a low potential in the antifungal activity. The presence of hydrophobic antifungal compounds is in agreement with Hernandez *et al*. (2007) who have reported antifungal activity in hydrophobic solvent-extracted compounds from *Cordia curassavica*. The activities of the most extracts were less than that of the control antifungal agent MCZ by at least 1 order of magnitude. *C. musae*, *F. oxysporum* and *S. rolfsii* were the highly affected fungi (MIC = 0.25-50, 0.5-50 and 0.25-25 mg mL^{-1}, respectively), in contrast with *A. flavus*, which was the least inhibited (MIC = 50-200 mg mL^{-1}), matching observations made by Iida *et al*. (1999). In concentrations lower than the MIC values, a slow growth of the fungi was observed in comparison to the controls.

MFC of Extracts

Ethyl acetate extraction was the most effective treatment, exhibiting an MFC from 10-50 mg mL^{-1}, while the extracts of 10% methanol in ethyl acetate and hexane exhibited MFC from 50-100 mg mL^{-1} and 100- >200 mg mL^{-1}, respectively (Table 2). These finding indicated that ethyl acetate extracts obtained from *S. aureofaciens* CMUAc130 culture performed a significant fungistatic and fungicide effect on different phytopathogenic fungi.

Antifungal Activity of Compounds 1-5

MIC values of isolated compounds 1-5 measured by the broth micro dilution method (Table 3). Compounds 1, 2 and 3 showed a total growth inhibition of the pathogenic fungi *F. oxysporum* at

Table 1: MIC of different extracts from culture of *S. aureofaciens* CMUAc130

	MIC[a] (mg mL^{-1})			
Test organisms	HE	EAE	10%M-EAE	MCZ
A. flavus	200	50.00	100	0.01
C. musae	50	0.25	25	0.01
P. ultimum	100	10.00	25	0.01
R. solani	100	5.00	10	0.01
F. oxysporum	50	0.50	25	0.01
S. rolfsii	25	0.25	10	0.01

[a]: MIC is defined as the lowest concentration providing complete inhibition of fungal growth. Results are the average of two replicates measured at 48 h after incubation. HE: hexane extract; EAE: ethyl acetate extract; 10%M-EAE: 10% methanol in ethyl acetate extract; MCZ: ethylenebis(dithiocarbamic acid) manganese zinc complex

Table 2: MFC of different extracts from culture of *S. aureofaciens* CMUAc130

	MFC[a] (mg mL^{-1})			
Test organisms	HE	EAE	10%M-EAE	MCZ
A. flavus	>200	100	100	0.01
C. musae	200	10	100	0.01
P. ultimum	200	50	100	0.01
R. solani	100	25	50	0.01
F. oxysporum	100	25	100	0.05
S. rolfsii	100	10	50	0.10

[a]: MFC is defined as the lowest concentration providing complete inhibition of fungal growth in a medium free of extract or compound. Results, the average of two replicates, were measured at 14 days after incubation. HE: hexane extract; EAE: ethyl acetate extract; 10%M-EAE: 10% methanol in ethyl acetate extract; MCZ: ethylenebis (dithiocarbamic acid) manganese zinc complex

Table 3: Antifungal activity of 1-5

Compounds	MIC[a] (mg mL^{-1})	Compounds	MIC[a] (mg mL^{-1})
1	0.30	4	10.000
2	1.00	5	20.000
3	0.40		
MCZ	0.025		

[a]: Results, the average of two replicates, were measured at 48 h from the beginning

0.30 (MIC 1), 1.00 (MIC 2) and 0.40 (MIC 3) mg mL^{-1}, respectively, while the MIC of compound 4 and 5 was observed at 10.0 (MIC 4) and 20.0 (MIC 5) mg mL^{-1}, respectively. The MIC value of compound 4 and 5 was high and produced low yield, so they were excluded for synergistic assay.

At 0.20 mg mL^{-1}, compound 1 developed 75% of growth inhibition (Fig. 2) while at 0.10 mg mL^{-1} showed a 25% inhibition. The same percentage of inhibition was detected when the compound 3 at 0.20 mg mL^{-1} was used. The fungal growth was inhibited by 20 and 60% when compound 2 at concentrations of 0.60 and 0.80 mg mL^{-1}, respectively was applied. As observed, the significant inhibitory activity of compounds 1-3 increased while the IC_{50} indicated a similar performance for 1 (0.17 mg mL^{-1}; 95% confidence interval = 0.16-0.43) and 3 (0.25 mg mL^{-1}; 95% confidence interval = 0.08-0.74) and this effect was better than that for 2 (0.78 mg mL^{-1}; 95% confidence interval = 0.37-1.24).

Because the resultant residue presented a higher antifungal activity (MIC and MFC = 0.50 mg mL^{-1}) than those exhibited by pure compounds, the presence of a synergistic effect between 1, 2 and 3 was suspected. The compounds were added in different combinations of two or three that in most cases were equivalent to half or less of their MIC values.

As shown in Table 4, when compound 1 at 0.15 mg mL^{-1} (0.5 MIC 1) was combined with 3 at 0.10 mg mL^{-1} (0.25 MIC 3) or with 2 at 0.50 mg mL^{-1} (0.5 MIC 2), a 100% inhibition in *F. oxysporum* growth was observed. The same results were obtained when the same concentration of 1, concentrations of 2 or 3 were tested and decreased to 0.25 mg mL^{-1} (0.25 MIC 2) and 0.04 mg mL^{-1} (0.1 MIC 3), respectively. When compound 1 was added at 0.075 mg mL^{-1}, equivalent

Table 4: Synergism among different concentrations of 1-3 combined in pairs

Compounds (mg mL^{-1})	Growth inhibition[a] (%)					
	2 (0.50)	2 (0.25)	2 (0.10)	3 (0.20)	3 (0.10)	3 (0.04)
1 (0.15)	100	100	80	100	100	100
1 (0.075)	90	70	37	95	80	75
1 (0.03)	75	50	20	80	60	45
3 (0.20)	85	62	40			
3 (0.10)	59	28	18			
3 (0.04)	35	10	3			

[a]Results, the average of two replicates, were obtained at 48 h from the beginning

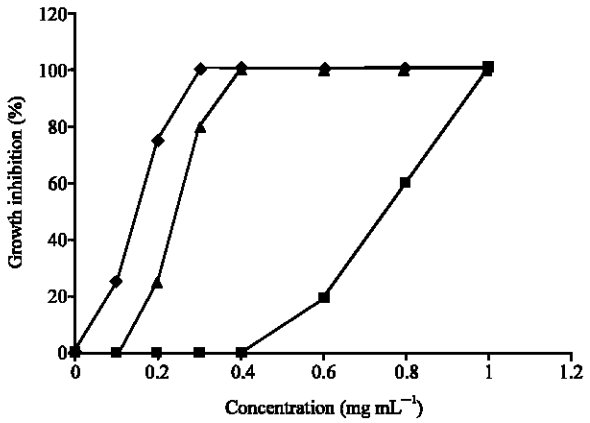


Fig. 2: Effect on fungi growth inhibition of different concentrations of 1 (◆), 2(■) and 3(▲)

to 0.25 of its MIC value and 2 at 0.50 mg mL^{-1}, a 90% inhibition was detected. When 1 was added at a concentration 10 times lower than the value of MIC 1 (0.03 mg mL^{-1}) and 2 at 0.50 mg mL^{-1} (0.5 MIC 2) or 3 at 0.20 mg mL^{-1} (0.5 MIC 3), a 75% and 80% inhibition were observed, respectively. When decreased the concentration of 2 to 0.25 mg mL^{-1} (0.25 MIC 2), a significant inhibition (50%) was still observed. When the same concentration of 1 was combined with 3 at 0.10 mg mL^{-1} (0.25 MIC 3), a 60% growth inhibition was shown when 3 was added at 0.04 mg mL^{-1} (0.1 MIC 3), the inhibition was 45%. These results indicated that the combination between 1 and 3 presented a poor synergistic effect than 1 and 2. Adding 2 and 3 at 0.50 (0.5 MIC 2) and 0.10 mg mL^{-1} (0.25 MIC 3), respectively, 59% inhibition was observed, reaching 85% when the concentration of 3 was 0.20 mg mL^{-1} (0.5 MIC 3). Lower concentrations of both compounds exhibited inhibition values ranging from 3 to 45%, meaning that the synergism between 2 and 3 is lower than that observed between 1 and 2 or between 1 and 3.

When all three compounds were combined together, each at a concentration of about 0.25 of its MIC, 100% control of the growth of *F. oxysporum* was obtained, demonstrating a better control than that observed when pairs of compounds were combined at approximately 0.25 of their MIC values. There was 63% inhibition on the fungal growth in 1 and 3 treatments at 0.03 (0.1 MIC 1) and 0.04 mg mL^{-1} (0.1 MIC 3), respectively and 2 at concentration of 0.10 mg mL^{-1} (0.1 MIC 3). If the concentration of at least one of the compounds was increased, the inhibition also increased, obtaining 88% inhibition when compounds 1 and 3 remained at the same concentration (0.1 MIC 1 and 3) and 2 was increased to 0.50 mg mL^{-1} (0.5 MIC 2) (Table 5).

In summary, when the antifungal active principles 1-3 were combined in pairs or all three together in concentrations below their MIC values, an unexpected fungal growth inhibition almost as high as that obtained with the synthetic compounds MCZ was observed. This result showed a clear

Table 5: Synergism among different concentrations of 1-3 combining all three

Compounds (mg mL^{-1})	Growth inhibition[a] (%)		
	2 (0.50)	2 (0.25)	2 (0.10)
1 (0.15)	100	100	100
3 (0.20)			
1 (0.075)	100	100	98
3 (0.10)			
1 (0.03)	88	75	63
3 (0.04)			

[a]: Results, the average of two replicates, were obtained at 48 h from the beginning

synergistic effect between them, which was not observed till now. This effect enabled the quantities of each compound needed for a total inhibition to be decreased at least 4-fold. The synergistic effect is one of the most important characteristics exhibited by natural extracts, increasing their efficacy in contrast to that which could be obtained with the equivalent amount of the active constituents alone (Amiguet *et al*., 2005; Cassella *et al*., 2002; Gershon *et al*., 2004; Hsu *et al*., 2007; Schultz and Nicholas, 2002; Tallarida, 2001; Yen and Chang, 2006).

In conclusion, this study demonstrates that extracts from the culture of *S. aureofaciens* CMUAc130 and their respective bioactive compounds showed an important fungitoxic effect against the microorganisms studied, increasing the antifungal potency of the active principles when they are combined.

ACKNOWLEDGMENTS

This study was supported of Faculty of Science, Silpakorn University, Nakorn Pathom, Thailand, as well as the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany (KIB), Chinese Academy of Sciences. The authors are grateful to Mr. Y.-N. He and Ms. H.-L. Liang in KIB for measuring NMR and MS data, respectively.

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