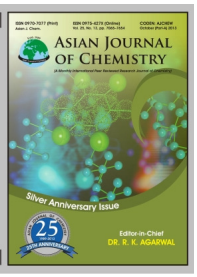
Asian Journal of Chemistry; Vol. 25, No. 17 (2013), 9667-9671

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15125



Inhibitory Effect of Extract from Twelve Chinese Medical Herbs and Two Active Quaternary Protoberberine Alkaloid from *Coptis chinensis Franch*

LONG CHEN[1], WEI ZHOU[1], LANG WU[1], MANLU GAO[1], TAIPING HOU[1,*] and KE TAO[2,*]

[1]Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, Sichuan University, Chengdu 610064, Sichuan Province, P.R. China
[2]College of Life Sciences, Sichuan University, Chengdu 610064, Sichuan Province, P.R. China

*Corresponding authors: Fax: +86 28 85214048; Tel: +86 28 85410992; E-mail: houtplab@scu.edu.cn

(*Received*: 10 January 2013; *Accepted*: 11 October 2013) AJC-14250

The traditional Chinese medical plants had been used for many centuries to support the immune system or to fight human infections. Among them, the phytochemicals of many herbs might suggest the possibility of their exploitations to the botany fungicides. In this study, twelve herbs used in the treatment of infectious diseases in traditional Chinese medicine were evaluated for *in vitro* activity against sixteen microbial plant pathogens. Among them, eleven were plant-pathogenic fungi and five were bacteria. All of the EtOH extracts were tested by the agar dilution technique. From the assessment of the crude extracts, the *Coptis chinensis Franch* were active against at least two microorganisms, especially to the *Valsa mali Miyabe et Yamada*. With the isolation and purification of *Coptis*, we finally obtained two pure antifungal active alkaloids *i.e.*, berberine and palmatine. And their resistances to the *Valsa mali Miyabe et Yamada* showed that the 50 % effective concentration (EC_{50}) of the berberine reached 9.84 ppm and the EC_{50} of palmatine was 243 ppm.

Key Words: Chinese medical herb, Antimicrobial, *Coptis chinensis Franch*, Berberine, Palmatine.

INTRODUCTION

The decrease of the agricultural production caused by plant pathogenic fungi or bacteria was one of the major economic damages in agriculture in China and other areas of the world. And it had been the biggest barrier to the development of the world-wide agriculture. Current strategies aimed at reducing crop losses rely primarily on chemical pesticides. However, most of the chemical pesticides were non-natural products or chemosynthesis compounds, which were efficient to the plant diseases and pests while it was also harmful to human. This strategies to control plant pathogenic microbial with classic pesticides may produce side effects[1], the use, overuse and misuse of pesticides in China have led to poisonings of farmers, degradation of land and water and increased levels of dangerous chemicals in China's food supply[2]. All of them has been shortened into one word "3R"(resistance, resurgence, residue). In recent years, the trend in agriculture towards greater sustainability and public concern for the "3R" questions associated with the use of chemical pesticides and fungicides has increased. Therefore, searching for a biological substitute, which is not only safe to human and environment but also efficient had become the priority issue of 21st century agriculture.

In recent decades, researches on botanical pesticides had shown to the world great progress, mainly because it was low-cost, abundant in resources, degradable and low-level-residue. Interests on the topic of antimicrobial plant extracts had been growing. Those so-called secondary metabolites of the herbs, such as tannins, terpenoids, alkaloids and flavonoids, were especially important and can protect plants against many microorganisms (virus, bacteria and fungi)[3-6]. Various spices and herb extracts had been used for the purpose of food preservation and appetizer promotion as well as medicinal purposes[3,4]. Among those herbs, traditional Chinese medicine (TCM), played a vital part in both ancient and modern Chinese medical system, had made a great contribution to modern therapeutics. From the studies of the traditional Chinese medicine, some of these medical plant extracts showing interesting antimicrobial activity were selected and extensively studied leading to the isolation and characterization of active constituents. For example, researches on *Erythrina crista* galli indicated that the herb contained several antimicrobial agents, pterocarpans and sandwicensin[7]. *Cinnamomum cassia* and *Curcuma longa* showed effective antifungal activities to the *S. choleraesuis*[8]. And the cucurmoschin of the black pumpkin seeds was also highly active *in vitro* against a wide range of fungi[9].

In this study, several Chinese medical herbs were selected to explore their agricultural usefulness. Most of them were commonly used in the traditional Chinese medical system and had demonstrated experimental and/or clinical antimicrobial and antiinfection effectiveness. Extract the samples with 95 % (v/v) ethanol in a hot water bath and the antiphytopathogenic microbial activities of crude extracts were evaluated using the Poisoned Food Technique[10]. The screening results of the antibacterial and antifungal activities showed the *Coptis chinensis Franch* and the *C. phellodendri* had efficient inhibition against all the fungi tested and exhibited strongest inhibitory effect against the *Valsa mali Miyabe et Yamada* and the *S. ampelinum*. From the better antifungal results and the separation and purification to the *Coptis chinensis Franch* EtOH crude extract, we eventually identified two pure and antifungal-active compounds berberine (Fig. 1) and palmatine (Fig. 2), which had never been reported the inhibition to any agricultural fungi. These two compounds should be useful pesticides candidates in the development of agricultural fungicides.

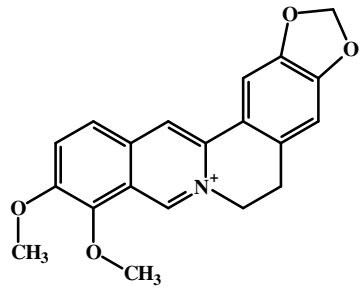


Fig. 1. Compound 1-berberine, $C_{20}H_{18}NO_4^+$

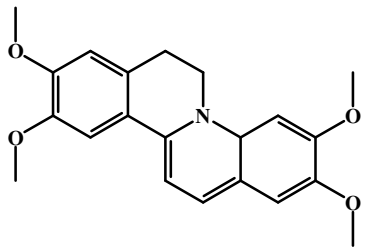


Fig. 2. Compound 2-palmatine, $C_{21}H_{22}NO_4^+$

EXPERIMENTAL

Twelve kinds of traditional Chinese medicines: *F. suspensa*; *So. Flavescens*; *A. capillaris*; *A. tataricus*, *H. cordata*, *Coptis chinensis Franch*; *A. asphodeloides*, *X. sibiricum*, *Herba taraxaci*, *C. phellodendri*, *Rh. Palmatum*, *S. baicalensis georgi* were all purchased from the Chengdu International Business Center, all specimen were identified by Dr Tao of Sichuan University. Eleven phytopathogenic fungi were maintained on potato dextrose agar (PDA: 20 % potato extract, 2 % dextrose and 1.8 % agar) and stored at 4 ± 1 ºC in the dark,Cultures of the five phytopathogenic bacteria were maintained on beef extract-peptone medium at 4 ± 1 ºC.

Spectroscopic analysis: ^1H, ^{13}C NMR spectra were measured at 27 ºC in DMSO-d_6 on Bruker AVII-400 MHz nuclear magnetic resonance apparatus. UV (in methanol/H_2O) was recorded on a Shimadzu SPDM10AVP photodiode detector (used as a part of RP-HPLC apparatus, 346 nm detected). ESI-MASS spectra were recorded on TSQ Quantum Ultra (Thermofish Scientific). HPLC was performed on a Shimadzu LC-06A, with the Intersil ODS-SP (5 μm × 4.6 mm × 150 mm) column.

Extraction and isolation: Twelve Chinese traditional medicine plants(dried already) were all crushed into powder (300 g) and soaked into the 95 % EtOH (2000 mL) at 45-50 ºC by the heating of thermostat water bath kettle. After the extraction, the ethanol extracts were filtered through the Whatman qualitative filter paper and then evaporated the liquid part to dryness *in vacuo* at 45 ºC, eventually yielding EtOH crude viscous extracts from different herb plant samples after removal of solvents.

Among the twelve Chinese traditional medicines, we selected the *Coptis chinensis Franch* powder (1000 g) to extract by the method above. The crude EtOH extract (200 g) of the *Coptis chinensis Franch* were all suspended with five times weight of pure water and then took 150 mL mix solvent partitioned with 3 times volumes of petroleum ether (PE), acetic ether and 1-butanol, yielding petroleum ether extract (15.3 g), ether acetic extract (4.0 g), 1-butanol extract (12.0 g) after removal of the solvent. The 1-butanol extract, a brown mass, was divided into a mass weighed 7 g and suspended in 3 mL methanol. Subjected to the column chromatography (500 g gel, 60 cm), flowed by gradient elution with trichloromethane in methanol (20/1, 10/1, 5/1, 2/1, 1/1, 0/1, v/v, 2000 mL per gradient) to yield six segments, based on the thin layer chromatography (TLC) analysis. Six segments were evaporated to dryness *in vacuo*, yield six masses (Fr1-6) of 464, 210, 534, 367, 373 and 1944 mg, respectively. Merging the Fr2,3,4 into one sample (1111 mg) and dissolved in small amount of methanol, applied to another column chromatography (100 g gel, 40 cm), eluted with $CHCl_3$/methanol/$(C_2H_5)_3N$ mix solvents (20:1:0.02-0:1:0.02,v/v/v), giving 5 fractions (Sr1-Sr5). By the analysis of the TLC under 346 nm UV detection and HPLC analysis, the Sr2 (97.5 mg), a yellow mass, contained two compounds which can both be detected under 346 nm. The Sr2 was dissolved into a small amount of methanol and applied to the TLC and then eluted by the mix solvents acetic ether/methanol (2/1, v/v), giving two fractions. HPLC analysis showed that the two fractions were pure compounds, compound **1** and compound **2**. Compound **1** and **2** showed a high purity of 93 and 95 %, respectively.

Bioassays: Twelve crude extracts of different herbs were tested against 11 phytopathogenic fungi: *F. graminearum*, *F. vasinfectum*, *P. aphanidermatum*, *G. candidum*, *S. ampelinum*, *Valsa mali Miyabe et Yamada*; *P. piricola*, *Alternaria alternaria* f.sp *mali*, *S. sclerotiorum*, *G. graminis*, *H. maydis*. The antifungal activity was determined by the plate growth method[11], using 1 % TWEEN-60's aqueous solution as the mix solvent at the concentration of 1 mg/mL. The various fungi were first incubated at 28 ºC for 72 h, the zones of the growth were measured and recorded at 28 ± 1 ºC when the growth cycle of negative controls was within the range of 50-70 mm. The zone of growth cycle was expressed as an average of the maximum diameter in four different directions. All tests were performed in triplicate. The fungi inhibition was calculated as the formula below:

$$I = \frac{(\overline{D}_1) - (\overline{D}_0)}{(\overline{D}_1)} \times 100\ \%$$

I is the inhibition rate, $\overline{D}_1$ is the average diameter of mycelia in the blank test and $\overline{D}_0$ is the average diameter of mycelia in the presence of fractions.

Furthermore, the original data only showed the inhibition of the antifungal activity unilaterally at several designated concentrations. The concentration for 50 % of maximal effect (EC_{50}) should also be calculated with the inhibition data from the concentration gradient of the same samples. The EC_{50} and their inhibition concentration-curves were all based on the methods B. Alexander's method[12] and calculated the data with EXCEL and SPSS.

A modification of the well diffusion assay protocol[13] was used to screen the twelve herbs' extracts for antibacterial activity against *S. aureus*, *Erwinia carotovora*, *Xanthomonas oryzae*, *Pseudomonas solanacea*, *Xanthomonas campestris*. The five bacteria were all cultured in the liquid medium for 16 h at 30 ºC and spread into the agar plates with ratio of 1:100 (v/v). Plant samples were dissolved in DMSO and diluted by 1 % TWEEN's water to the final concentration of 1 mg/mL (1000 ppm) and sterilized by the filtration trough the 0.45 μm sterilizing Millipore filter. Wells were made in agar plates using the broad end of a sterile Pasteur pipette (8 mm diameter) and 120 μL extract solutions was added to each well. Pure water was used as negative control. Streptomycin (200 μL/mL) for agriculture use was used as the positive reference standards to determine the sensitivity of each microbial species tested. The inoculate plates were incubated in 30 ºC for 24 h. Antibacterial activity was evaluated by measuring the diameter of inhibition zone of the test bacteria. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Antibacterial activities of twelve herb extracts: The results of screening the antibacterial activities of 12 herb extracts were given on Table-1. Among those herb's extracts, only the *Coptis chinensis Franch* and the *C. phellodendri* showed little antibacterial activities to the *S. aureus* and the *Xanthomonas campestris*, while the positive control streptomycin showed strong resistance to all the bacteria tested.

Antifungal activities of 12 herb extracts: The results of screening the antifungal activities of 12 herb's extracts were displayed on Table-2A-B. Among all the specimens, the *Coptis chinensis Franch* showed great resistance to several phytopathogenic fungi.

***Coptis chinensis Franch* isolation and bioassays:** The 12 herb extracts' screening experiments for the antifungal activities showed the *Coptis chinensis Franch* had a vital inhibition to the *Valsa mali Miyabe et Yamada*. And the further partition offered us the 1-butanol part, which the value of EC_{50} was 34.4 ppm and the EC_{90} was 233 ppm (NORMSINV(y) = 2.63 + 0.67 × LN(x)-5, R^2 = 0.8512). And the first column chronographic segments Fr2.3.4 combination showed the inhibition to the *Valsa mali Miyabe et Yamada*, the EC_{50} was 13 ppm, the EC_{90} was 174 ppm (NORMSINV(y) = 3.73 + 0.49 × LN(x)-5, R^2 = 0.8800). And through TLC chromatography, the second column chronographic segments Sr2 separated 2 highly-pure compounds, compound **1** and **2**. Their resistances to the *Valsa mali Miyabe et Yamada* were also tested. The compound **1** indicated the EC_{50} reached the concentration of 9.84 ppm (NORMSINV(y) = 3.14 + 0.81 × LN(x)-5, R^2 = 0.9972). And the compound **2** indicated a little less-effective inhibition, its EC_{50} was 243 ppm (NORMSINV(y) = 2.77 + 0.41 × LN(x) - 5, R^2 = 0.8789).

Spectrum data: The compound **1** and **2** were all characterized under 346 nm UV detection and HPLC analysis. Both of them were yellow mass, easily dissolved in methanol.

The ESI-mass detection of compound **1** exhibited anion at molar mass 336.05. The ^1H and ^{13}C NMR data of compound **1** (400 Mz, DMSO-d_6) was given in Table-3.

The ESI-mass detection of compound **2** exhibited anion at molar mass 352.05. The ^1H and ^{13}C NMR data of compound **2** (400 Mz, DMSO-d_6) was given in Table-4.

According to the bioactivities of 12 Chinese medical herbs extracts, all of them showed little resistance to the phytopathogenic bacteria, only the *Coptis chinensis Franch* and the *C.*

TABLE-1
ANTIBACTERIAL ACTIVITIES OF 12 HERBS

Herbs	Bacterial species				
	S. aureus	*Erwinia carotovora*	*Xanthomonas oryzae*	*Pseudomonas solanacea*	*Xanthomonas campestris*
Coptis chinensis Franch	++	–	–	–	++
A. asphodeloides	–	–	–	–	–
F. suspensa	–	–	–	–	–
So. flavescens	–	–	–	–	–
A. tataricus	–	–	–	–	–
H. cordata	–	–	–	–	–
A. capillaris	–	–	–	–	–
C. Phellodendri	++	–	–	–	–
Herba taraxaci	–	–	–	–	–
X. sibiricum	–	–	–	–	–
Rh. Palmatum	–	–	–	–	–
S. baicalensis georgi	–	–	–	–	–
Streptomycin (positive control)	++++	++++	++++	++++	++++

Table-1: ++++ represents the diameter of zone of inhibition was between 20-25 mm, which only the Streptomycin gave to all tested bacteria. ++ represents the diameter of zone of inhibition was between 10-15 mm. – represents the diameter of zone of inhibition was almost 0 mm, which indicted the herb extracts showed no inhibition to the bacteria tested.

TABLE-2A-B
ANTIFUNGAL ACTIVITIES OF 12 HERB EXTRACTS AGAINST 11 PHYTOPATHOGENIC FUNGI.
THE PERCENTAGES WERE THE INHIBITION RATE OF THE DIFFERENT HERBS AGAINST DIFFERENT FUNGI.
THE CONCENTRATION OF THE HERB EXTRACTS WAS SETTLED AT 1 mg/mL (1000 ppm)

A

Fungi species	Herbs					
	Coptis chinensis Franch (%)	*A. asphodeloides* (%)	*F. suspensa* (%)	*So. flavescens* (%)	*A. tataricus* (%)	*H. cordata* (%)
P. piricola	0.00	35.93	10.37	14.17	17.08	15.36
Alternaria alternaria f.sp mali	43.59	8.82	23.42	30.53	28.77	13.27
Valsa mali Miyabe et Yamada	87.10	71.52	73.64	7.56	39.86	25.33
F. graminearum	37.41	17.79	21.74	15.02	36.94	22.05
G. candidum	26.50	5.93	2.82	2.97	3.96	-0.99
P. aphanidermatum	7.99	20.41	23.26	14.13	36.80	32.06
S. sclerotiorum	5.62	5.21	3.47	4.30	10.75	5.13
S. ampelinum	99.19	48.43	38.36	31.05	36.47	33.97
F. vasinfectum	33.62	60.32	60.95	17.09	16.52	17.50
G. graminis	6.94	4.86	2.78	4.86	9.38	-0.35
H. maydis	28.01	16.31	20.92	9.57	14.54	11.35

B

	A. capillaris (%)	*C. phellodendri* (%)	*Herba taraxaci* (%)	*X. sibiricum* (%)	*Rh. Palmatum* (%)	*S. baicalensis georgi* (%)
P. piricola	6.74	30.27	40.48	27.89	11.71	39.94
Alternaria alternaria f.sp mali	14.97	34.60	18.73	18.10	27.97	24.14
Valsa mali Miyabe et Yamada	19.00	85.11	31.91	2.48	38.72	69.02
F. graminearum	0.00	31.90	11.47	8.24	9.14	27.15
G. candidum	0.99	3.96	5.61	-1.98	1.67	21.33
P. aphanidermatum	5.08	9.69	17.66	6.27	2.78	40.56
S. sclerotiorum	17.09	8.70	48.99	4.93	3.79	35.86
S. ampelinum	27.24	85.58	38.46	12.82	22.50	46.67
F. vasinfectum	13.89	23.81	17.26	4.17	9.12	36.75
G. graminis	-2.78	-13.89	-5.56	2.08	-11.46	5.90
H. maydis	16.67	21.63	21.28	2.84	20.92	34.75

TABLE-3
^{1}H NMR AND ^{13}C NMR DATA OF COMPOUND **1** (δ IN ppm)

Proton No.	^{1}HNMR signal	Proton No.	^{13}CNMR signal
H-5	3.21(t, *J*=6.0Hz,2H)	C-9	150.8 (1C, C)
H-5	3.35(t, *J*=6.0Hz,2H)	C-3	150.2 (1C, C)
9, 0-OCH_3	4.10(s,6H)	C-2	148.1 (1C, C)
H-6	4.95(t, *J*=6.0Hz,2H)	C-8	145.9 (1C, CH)
2,3-OCH_2O	6.18(t,2H)	C-10	144.1 (1C, C)
H-4	7.09(s,1H)	C-13a	137.9 (1C, C)
H-1	7.80(s,1H)	C-12a	133.5 (1C, C)
H-12	8.00(d, *J*=9.1Hz,1H)	C-4a	131.1 (1C, C)
H-11	8.21(d, *J*=9.1Hz,1H)	C-13	127.2 (1C, CH)
H-13	9.00(s,1H)	C-12	124.0 (1C, CH)
H-8	9.91(s,1H)	C-13b	121.8 (1C, C)
–	–	C-11	120.9 (1C, CH)
–	–	C-8a	120.7 (1C, C)
–	–	C-4	108.9 (1C, CH)
–	–	C-1	105.9 (1C, CH)
–	–	O-CH_2-O	102.5 (1C, CH_2)
–	–	(C_9-)OCH_3	62.4 (1C, CH_3)
–	–	C-6	57.5 (1C, CH_2)
–	–	C_{10}-OCH3	55.6 (1C, CH_3)
–	–	C-5	26.8 (1C, CH_2)

TABLE-4
^{1}H NMR AND ^{13}C NMR DATA OF COMPOUND **2** (δ in ppm)

Proton No.	^{1}H NMR signal	Carbon No.	^{13}C NMR signal
H-5	3.23 (t, *J* = 6.0 Hz, 2H)	C-9	151.4 (1C, C)
-OCH_3	3.87 (s, 3H)	C-3	150.2 (1C, C)
-OCH_3	3.94 (s, 3H)	C-2	148.6 (1C, C)
-OCH_3	4.07 (s, 3H)	C-8	145.4 (1C, CH)
-OCH_3	4.10 (s, 3H)	C-10	143.6 (1C, C)
H-6	4.97 (t, *J* = 6.0 Hz, 2H)	C-13a	137.6 (1C, C)
H-4	7.09 (s, 1H)	C-12a	133.8 (1C, C)
H-1	7.73 (s, 1H)	C-4a	128.5 (1C, C)
H-12	8.06 (d, *J* = 9.4 Hz, 1H)	C-13	126.7 (1C, C)
H-11	8.19 (d, *J* = 9.4 Hz, 1H)	C-12	123.4 (1C, CH)
H-13	9.12 (s, 1H)	C-13b	121.3 (1C, C)
H-8	9.91 (s, 1H)	C-11	119.9 (1C, CH)
–	–	C-8a	118.9 (1C, C)
–	–	C-4	111.2 (1C, CH)
–	–	C-1	108.7 (1C, CH)
–	–	C-6	61.9 (1C, CH_2)
–	–	C10-OCH_3	57.0 (1C, CH_3)
–	–	C9-OCH_3	56.1 (1C, CH_3)
–	–	C3-OCH_3	55.8 (1C, CH_3)
–	–	C2-OCH_3	55.3 (1C, CH_3)
–	–	C-5	25.9 (1C, CH_2)

Phellodendri indicated effectiveness to two phytopathogenic fungi, *Valsa mali Miyabe et Yamada* and *S. ampelinum*. The inhibition rate of the *Coptis chinensis Franch* against *Valsa mali Miyabe et Yamada* was 87.10 % and the inhibiton rate against *S. ampelinum* was 99.9 %. The *C. phellodendri* was also effective to *Valsa mali Miyabe et Yamada* and *S. ampelinum* (the inhibition rates were 85.11 and 85.58 %, respectively).

Bioactivities of the crude extracts guided the isolation of the *Coptis chinensis Franch*, which led to the discovery of compounds **1** and **2**, which were conclusively identified to be protoberberine alkaloids. On the basis of extensive 1D and 2D NMR spectroscopic analysis and the ESI-MS analysis, the

compound **1** was in accordance with the molecular formular of $C_{20}H_{18}NO_4^+$ (berberine). The ^1H and ^{13}C NMR data matched with the berberine's ^1H and ^{13}C NMR data[14,15]. And ESI-MS data and the UV detection of the berberine inhibited the same peak with the compound **1**. We could draw the conclusion that the compound **1** was the known yellow quaternary protoberberine alkaloid berberine[16] (Fig. 1). Its molecular formula was $C_{20}H_{18}NO_4^+$. Its absolute configuration is 5,6-dihydro-9,10-dimethoxy-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium.

The compound **2** was in accordance with the molecular formula of $C_{21}H_{22}NO_4^+$ (palmatine). The ^1H and ^{13}C NMR data matched with the palmatine's ^1H and ^{13}C NMR data[15]. And ESI-MS data and the UV detection of the palmatine inhibited the same peak with the compound **2**. We could draw the conclusion that the compound **2** was the known yellow quaternary protoberberine alkaloid palmatine[16] (Fig. 2). Its molecular formula was $C_{21}H_{22}NO_4^+$. Its absolute configuration is 5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium.

With the isolation and the purification of the *Coptis chinensis Franch*, the inhibition rates of different part of the isolation products against to the *Valsa mali Miyabe et Yamada* has been calculated by the methods Alexander *et al.* method[12]. The EC_{50} of each part of the *Coptis chinensis Franch* inhibited that its EC_{50} was always under 50 ppm. Moreover one of the monomers obtained (berberine) showed the highest inhibition and the value of EC_{50} reached the concentration of 9.84 ppm. At present it is not comparable to pesticides in meeting efficacy, market and other expectations[17], but it still has a potential to be developed as a new sort of environment-friendly antifungal drug in the agricultural chemistry field. Further studies on confirming the *in vivo* efficacy of extract and the application of compounds **1** and **2** are undergoing in our laboratory.

ACKNOWLEDGEMENTS

This study is supported by the National Natural Science Foundation of China (No. 31272068/C140501) and Hi-tech Research and Development Program of China (863 Program, No. 2011AA10A202-3) and National Key Technology Research and Development Program of the Ministry of Science and Technology of China (2011BAE06B01-23).

REFERENCES

1. M.T. McGrath, *Plant Dis*., **85**, 236 (2001).
2. J.K. Huang, F.B. Qiao, L.X. Zhang and S. Rozelle, Farm Pesticide, Rice Production and Human Health, EEPSEA Working Paper, EEPSEA, Singapore (2000).
3. M.M. Cowan, *Clin. Microbiol. Rev*., **10**, 564 (1999).
4. G.C.L. Ee and Y.L. Cheow, *Asian J. Chem.*, **20**, 343 (2008).
5. L. Zaika, *J. Food Safety*, **9**, 97 (1988).
6. M. Wink, *Theor. Appl. Genet*., **75**, 225 (1988).
7. L.A. Mitscher, S.R. Gollapudi, D.C. Gerlach, S.D. Drake, E.A. Veliz and J.A. Ward, *Phytochemistry*, **27**, 381 (1988).
8. S.-H. Lee, K.-S. Chang, M.-S. Su, Y.-S. Huang and H.-D. Jang, *Food Control*, **18**, 1547 (2007).
9. H.X. Wang and T.B. Ng, *Peptides*, **24**, 969 (2003).
10. R.K. Grover and J.D. Moore, *J. Phytopathol*., **52**, 876 (1962).
11. L. Zhou, F. Gao, Y. Teng and T.P. Hou, *J. Sichuan Univ*., **45**, 214 (2008). (Nat Sci Ed).
12. B. Alexander, D.J. Browse, S.J. Reading and I.S. Benjamin, *J. Pharmacol. Toxicol. Methods*, **41**, 55 (1999).
13. H.R. Juliani Jr., F. Biurrun, A.R. Koroch, M.M. Oliva, M.S. Demo, V.S. Trippi and J.A. Zygadlo, *Planta Med*., **68**, 762 (2002).
14. M.E. Mastafa and Q.H. Ahmad, *J. Heterocycl.*, **27**, 912 (1988).
15. Q. Minjian and W. Hengqi, *Chem. Ind. Forest Prod*., **12**, 42 (2003).
16. S. Man, M. Potácek, M. Necas, Z. •ák and J. Dostál, *Molecules*, **6**, 433 (2001).
17. B. Gerhardson, *Trends Biotechnol*., **20**, 338 (2002).