

Full Length Research Paper

Separation and determination of the bioactivity of oosporein from *Chaetomium cupreum*

Bi-Zeng Mao^{1#}, Chao Huang^{1#}, Guan-Mei Yang¹, Yu-Zhe Chen² and Shao-Yuan Chen^{1*}

¹State Key Laboratory for Rice Biology, Institute of Biotechnology, Zhejiang University, Huajiachi Campus, Hangzhou, Zhejiang, 310029, People's Republic of China.

²College of Pharmaceutical Science, Zhejiang University, Hangzhou, People's Republic of China.

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Many plants have devised methods of protecting themselves; one of such methods is the use of endophytic fungi. The antagonistic mechanism by endophytic fungi has rarely been revealed. This study investigated *Chaetomium cupreum* from *Macleaya cordata* (Willd.) R.Br Herb, which was identified by analysis of morphological characteristics and 28S rDNA sequence. The crystal isolated from culture broth of *C. cupreum* was identified by X-ray single crystal diffraction as 3,3',6,6'-tetrahydroxy-4,4'-dimethyl-1,1'-bi(cyclohexa-3,6-diene)-2,2',5,5'-tetraone (abbreviated as oosporein). We demonstrated that oosporein had antifungal activity against *Rhizoctonia solani*, *Botrytis cinerea*, *Pytium ultimum*, and antitumor activities against HL-60 and A549. It is toxic to *Artemia salina*.

Key words: *Chaetomium cupreum*, toxicity, antifungal activity, oosporein, antitumor.

INTRODUCTION

Natural products could be discovered from nature and these are important ways of getting new drugs. Endophytic fungi can be found in every plant species, and they appear to contribute significantly to fungal bioactivity (Arnold et al., 2001). They can be used as an alternative host for medicinal activity (Lei, 2008). Endophytic fungi are antagonistic to plant pathogens and even to some plant pathogenic fungi (Samuels et al., 2006), and they are also capable of biodegrading poly aromatic hydrocarbons (PAHs) (Harrison Ifeanyichukwu, 2009). Now, research has shown that *Chaetomium fusiforme* fungus has displayed a wide range of antimicrobial and antitumor activities (Lei, 2008), and some endophytic fungal pigments are aimed towards antagonizing human pathogenic bacteria (Visalakchi and Muthumary, 2009). Metabolites are the main reason why the endophytic fungi showed different bioactivity, according to their bioactivity and diversity, and acted as a supply of new structural type model for development of drugs.

Our study isolated oosporein from the metabolites of

endophytic fungi, *Chaetomium cupreum*. *C. cupreum* is an endophytic fungus from *Macleaya cordata* (Willd.) R.Br Herb belonging to Papaveraceae family located in the northwest district of Zhejiang Province, China, and is known to produce alkaloids (Dang et al., 2009). The red oosporein, a widespread metabolite of soil-dwelling fungi (Strasser, 2000), showed moderate antibiotic (Brewer, 1984; Strasser, 2000) and antifungal activities against *Phytophthora infestans* and some other phytopathogenic fungi (Toshinori et al., 2004). We demonstrated that oosporein is capable of antagonizing *Rhizoctonia solani*, *Botrytis cinerea* and *Pytium ultimum*, having antitumor activity against HL-60 and A549 and acute toxicity against *Artemia salina*.

MATERIALS AND METHODS

Isolation and identification of the endophytic fungal strain ZJWCF079

C. cupreum ZJWCF079, isolated from a twig of *Macleaya cordata*, was collected in Zhejiang Province of the People's Republic of China, and maintained in the China Center for Type Culture Collection under the number CGMCC 3110. The strain ZJWCF079 was identified by analyzing the morphological characters according to Pornsuriya et al. (2008). Also, sequence analysis of the 28s rDNA was done using the primer pair LROR and LR5 (Moncalvo et al., 2002; Chu-Long, et al.,

*Corresponding author. E-mail: chensy@zju.edu.cn.

These authors contributed equally to this work.

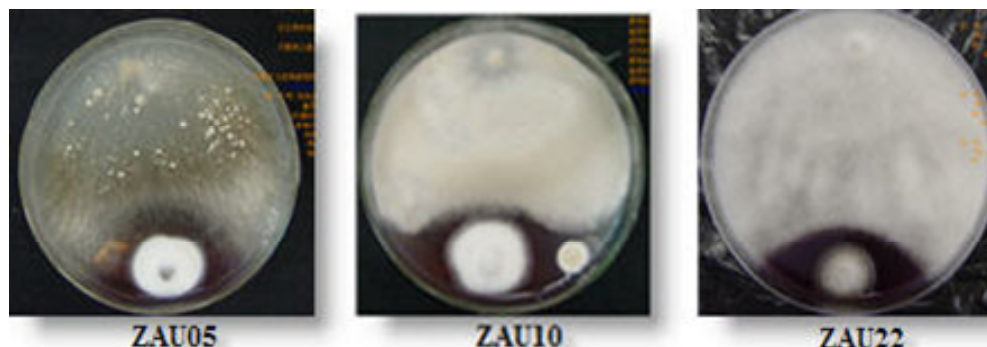


Figure 1. Identification of the antifungal activity against ZJUP05, ZJUP10 and ZJUP22.

2008).

Determination of antifungal activity of *C. cupreum* ZJWCF079 and cumulative effect

The fungistatic activity of *C. cupreum* was tested in the potatodextrose agar (PDA) medium against three plant pathogenic fungi: *R. solani* (strain ZJUP05), *B. cinerea* (strain ZJUP10), and *P. ultimum* (strain ZJUP22) - all the numbers refer to the collection center at the Fungal Laboratory of Zhejiang University. A block of the growing surface of the strain ZJWCF079 (5 mm diameter), which was pre-grown on PDA, was placed on the edge of a dish. The dish was incubated at 25°C for 3 days, after which a block of the growing surface of the plant pathogenic fungus was placed on the opposite position against *C. cupreum* ZJWCF079, then the growth of both was observed (Chu-Long et al., 2008; Adewusi and Afolayan, 2009). The experimental steps were repeated and incubation time was changed from four to eight days.

Liquid culture of *C. cupreum* ZJWCF079 and determination of antifungal activities of culture broth

C. cupreum ZJWCF079 was grown in surface culture in 1000-ml Erlenmeyer flasks containing 400 ml of PDB (liquid medium of PDA) medium, which consists of potato (200 g/l) and glucose (20 g/l). The flasks were incubated with shaking at 150 rpm/m at 25°C. After four days, the culture broth was filtrated by bacteria filter, and then two kinds of concentration (10 and 20%) of the culture broth were diluted with melted PDA medium and poured into a Petri dish. After solidification, a disk of the growing surface of the pathogenic fungus (5 mm diameter), containing ZAU05 and ZAU22 pre-grown on PDA medium, was located in the center of the dish. The strains ZAU22 and ZAU05 were incubated for 3 days.

The experimental steps were repeated, and incubation time was changed from five to ten days (Chu-Long et al., 2008).

Isolation of antifungal metabolites from *C. cupreum* ZJWCF079 culture broth

The culture broth was extracted with an equal volume of ethyl acetate. The ethyl acetate phase was filtered and evaporated *in vacuo*. Samples were then re-dissolved in trichloromethane, and filtered to leave mixtures. Mixtures were re-dissolved in mixed solvent (distilled water: methanol 1:1, v/v), and also filtered to remove solid particles and extracted with enough ethyl acetate. The ethyl acetate phase was evaporated *in vacuo* to get the saturated solvent. The saturated solvent was put at 4°C, and red single crystal was obtained.

Chemical analysis and determination of toxicity and antitumor activity of crystal

The crystal was analyzed by single crystal diffraction (Crystal structure 3.7.0), and chemical and spatial structure was determined. *A. salina* was used as test organism for assessment of acute toxicity of the chemical crystal (Svensson et al., 2005). The crystals did not dissolve in distilled water, but in dimethylsulfoxide (DMSO). Its mass concentrations of 20, 10, 6 and 2 ppm were prepared as series of mother liquor in 10% DMSO distilled water solution. Artificial sea water and mother liquor, in accordance with volume ratio of 1:1 were mixed. The final concentrations were 10, 5, 3 and 1ppm. Podophyllotoxin was prepared as positive control, and distilled water as negative control. HL-60 and A54 were used as a model for testing antitumor activity and VP-16 was used as a positive control.

RESULTS

Identification of the strain ZJWCF079 as *C. cupreum*

When strain ZJWCF079 grew on the PDA medium, it formed pure white colonies. The medium was red due to a red pigment exudate, maturing within 7 - 10 days on PDA medium of ascomatal hairs arcuate, apically circinate or coiled septate (Pornsuriya et al., 2008). The strain grew slowly, daily radial expansion of growth rate was 3 mm/d without producing any spores, and a large number of red pigment exudates were easily generated, which had the characteristics of *C. cupreum*. To verify result by molecular analysis, 28s rDNA regions were amplified and sequenced. The 28s sequence was known to be diagnostic in endophytic fungi (Suyanto et al., 2003). A blast research of the resulting sequence against GenBank identified it to be 100% identical to the *C. cupreum* strains MUCL 10178 (AF286400). Therefore, it could be concluded that the strain ZJWCF079 is an isolate of *C. cupreum*.

Determination of antifungal activity of *C. cupreum* ZJWCF079 and culture broth

To prove the antifungal activity, the strain ZJWCF079 and plant pathogenic fungi (ZJUP05, ZJUP10 and ZJUP22) were grown at the opposing point on the PDA medium.

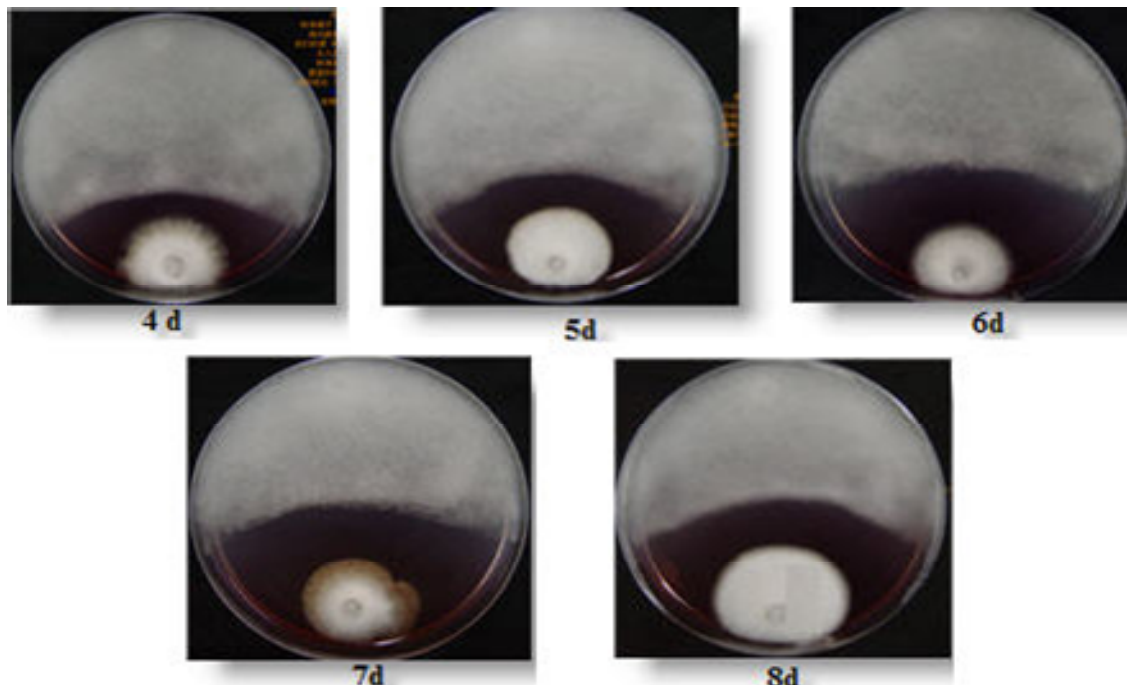


Figure 2. Identification of the cumulative effect of metabolites.

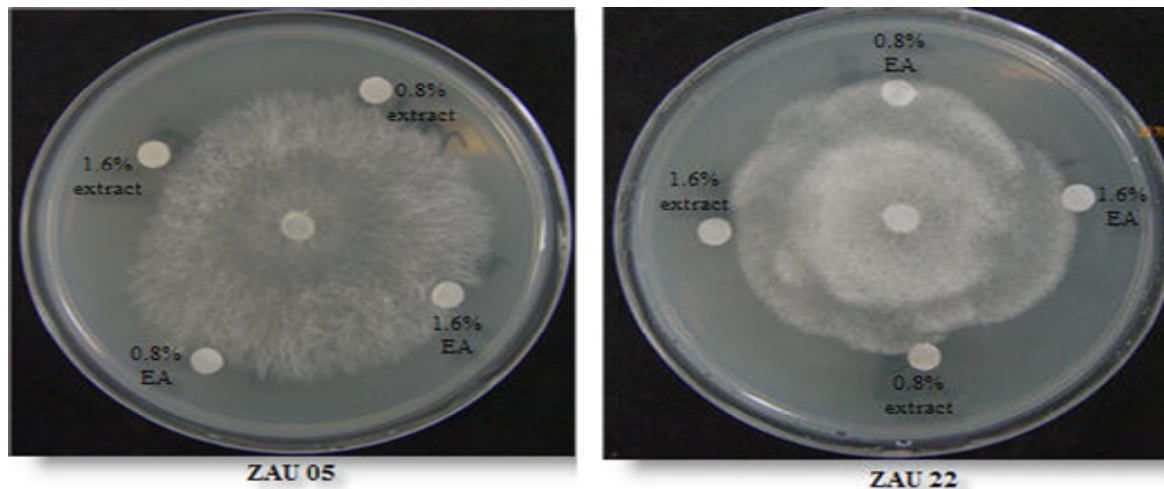


Figure 3. Identification of the cumulative effect of metabolites.

After three days, the result was shown in Figure 1 and cumulative effect, with regards to the passing of time, was shown in Figure 2; the longer the incubation period the longer the inhibition zone. Metabolites were accumulated in gel, and inhibited growth of ZAU22 and ZAU05. The inhibition zone gel was cut off, mashed and soaked in ethyl acetate for two days. After filtration, the ethyl acetate phase was evaporated *in vacuo*. Ethyl acetate was used to re-dissolve the extract. Two kinds of concentration of the extract were put in filter paper (5 mm diameter) which was sterilized and kept 2 cm away from the center. EA was used as blank control. The strains, ZJU05 and ZJU22,

were put in the center and incubated for 2 days. The result is shown in Figure 3.

Liquid culture of *C. cupreum* ZJWCF079 and determination of antifungal activities of culture broth

The antifungal activity results of liquid culture are shown in Figures 4 and 5. As shown in Figures 4 and 5, there was growth of the strains, ZAU05 and ZAU22, on mixture PDA medium as well as blank control. The antifungal ingredients in metabolites would change over time.

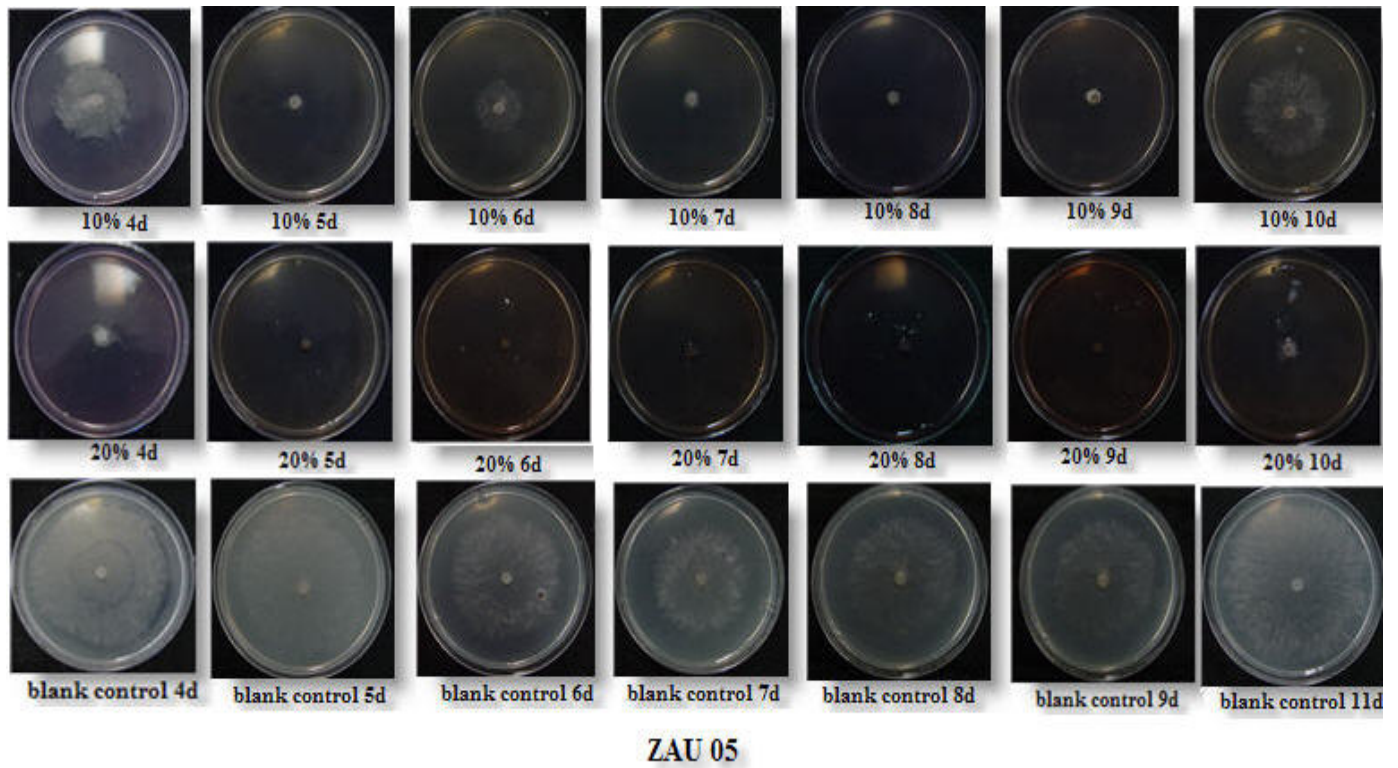


Figure 4. The growth of ZAU05 on mixture medium as well as blank control.

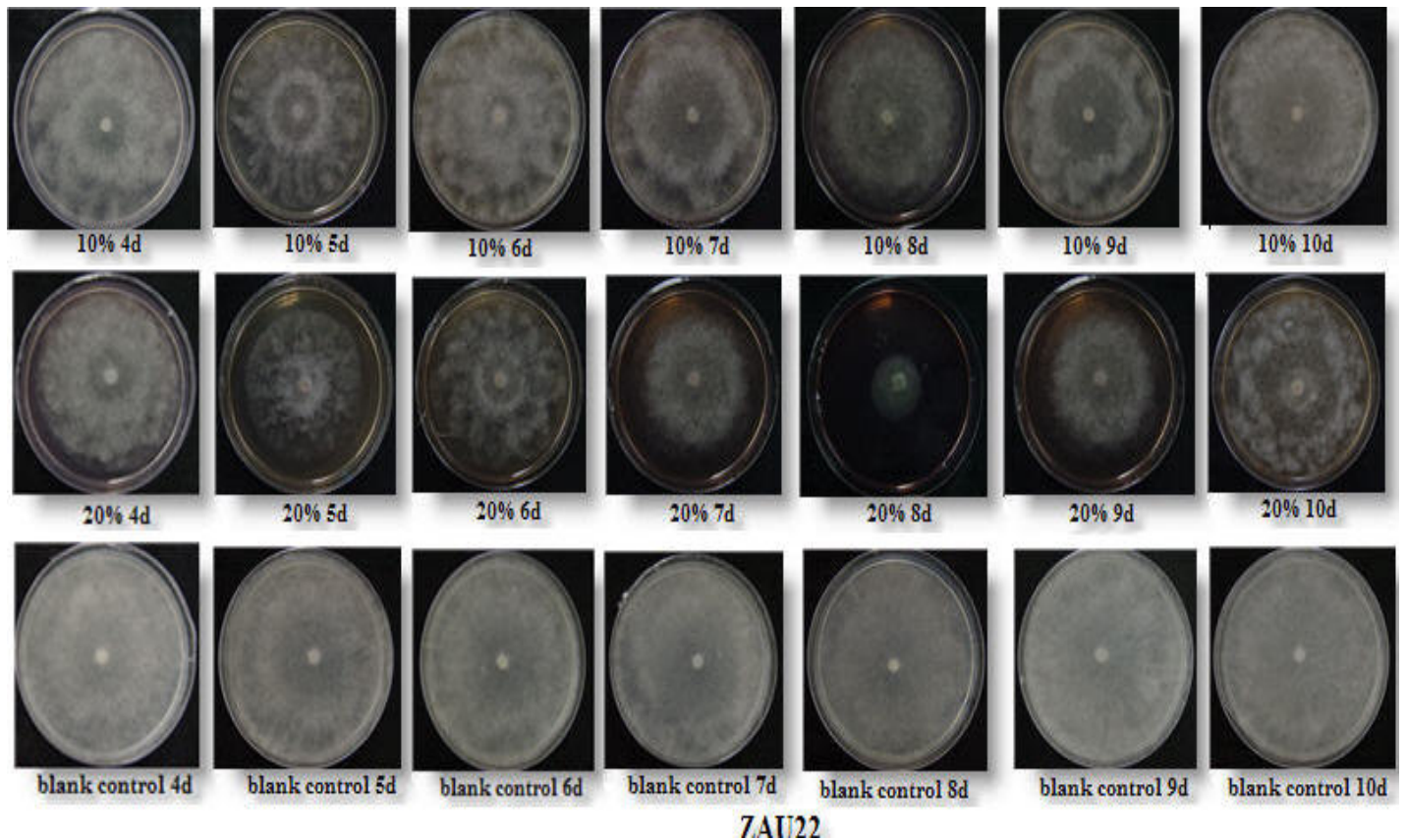


Figure 5. The growth of ZAU22 on mixture medium as well as blank control.

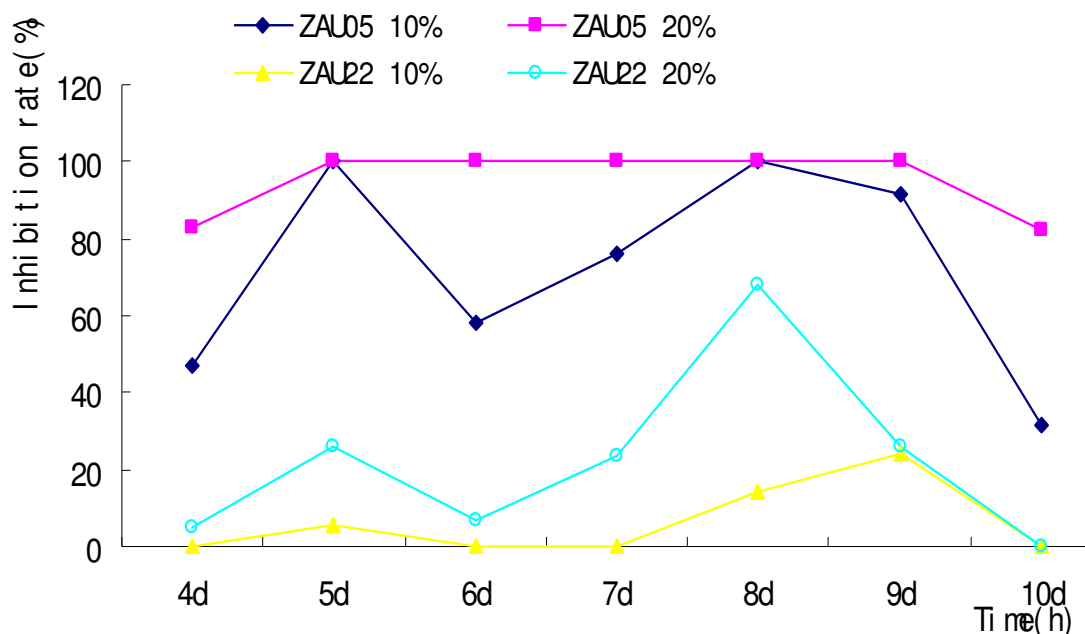


Figure 6. Changes of antifungal ingredients in metabolites over time.

According to the record, we converted the photo data to curve diagram, as shown in Figure 6.

Determination of crystal structure

The crystal structure was analyzed and determined by single crystal diffraction. The selected important data are given in Table 1. The compound was red crystals, its melting point was 248~250 °C (decomposition), and it was difficult to dissolve in water but was soluble in ethyl acetate. Molecular weight was 306 and molecular formula was $C_{14}H_{10}O_8$. The chemical name is 3, 3',6,6'-tetrahydroxy-4,4'-dimethyl-1,1'-bi(cyclohexa-3,6-diene)-2,2',5,5'-tetraone, oosporein for short.

Evaluation of crystal toxicity

A. salina was used to evaluate toxicity of oosporein. The results are given in Table 2. It was shown that oosporein had great toxicity against *A. salina*. Calculating corrected mortality, the number of dose-mortality probability method was used to get a half inhibitory concentration ($LC_{50} = 1.0 \mu\text{g/ml}$). Oosporein was demonstrated to have great acute toxicity as well as podophyllotoxin by *A. salin* larvae test.

Determination of antitumor of crystal

Result of antitumor activity test was given in Table 3. Oosporein processed some antitumor activity, but not as well as VP-16.

DISCUSSION

Chaetomium belongs to ascomycetes, chaetomiaceae, and is the largest saprophytic-ascomycetes genera (Somdej et al., 2006). *C. cupreum* is a widely distributed soil fungus that antagonizes numerous fungal phytopathogens. It has the potential of being a biocontrol agent against a range of plant pathogens on the basis of diverse metabolites, mycoparasitism, competition for space and nutrients, or various combinations of these. Some species of these are good cellulase and laccase producers, and are used in biotechnological industry (Ankudimova et al., 1999; Mimura et al., 1999); also, some of these produce cell-wall-degrading enzymes, such as chitinases (Inglis and Kawchuk, 2002).

In our study, the strain ZJWCF079 was identified as *C. cupreum*, and had effect on plant pathogenic fungi, such as *R. solani*, *B. cinerea* and *P. ultimum*. As shown in Figure 1, the inhibition zone meant that strain ZJWCF07 has secreted some active substances of anti-pathogen along the gel that inhibited the growth of pathogenic fungi. Then inhibition zone gel was dug and soaked in ethyl acetate. The concentrated ethyl acetate phase showed antifungal activity as shown in Figure 3, and active substance of anti-pathogen accumulated in gel as shown in Figure 2. Liquid culture was fit to large-scale fermentation, the activity of culture broth had been verified as shown in Figures 4 and 5, and this would change over time as shown in Figure 5. When the fermentation time was 8 days, inhibition rate reached the maximum (Inhibition rate of ZAU05 on 10% mixed medium was 100%, ZAU05 of 20% mixed medium was 100%, ZAU22 of 10% mixed medium was 14.1% and ZAU22 of 20% mixed medium was 68.2%).

Table 1. Selected important data about oosporein.

Bond	Distance (Å)	Bond	Distance (Å)	Bond	Distance (Å)
O(1)–C(3)	1.222(2)	C(2)–C(7)	1.341(2)	O(4)–H(4)O(1)	1.896
O(2)–C(4)	1.3230(16)	C(3)–C(4)	1.509(2)	C(1)–H(1)	0.960
O(3)–C(6)	1.2220(19)	C(4)–C(5)	1.350(2)	C(1)–H(2)	0.960
O(4)–C(7)	1.3342(17)	C(5)–C(6)	1.4535(17)	C(1)–H(3)	0.960
C(1)–C(2)	1.4999(19)	C(6)–C(7)	1.5103(2)		
C(2)–C(3)	1.4503(19)	O(2)–H(2)O(1)	0.897		
Angle	(°)	Angle	(°)	Angle	(°)
C(1)–C(2)–C(3)	118.99(14)	C(4)–C(5)–C(5)	122.45(11)	C(4)–O(2)–H(2)O(1)	110.5
C(1)–C(2)–C(7)	123.75(13)	C(4)–C(5)–C(6)	117.98(11)	C(7)–O(4)–H(4)O(1)	111.8
C(3)–C(2)–C(7)	117.26(12)	C(5)–C(5)–C(6)	119.56(12)	C(2)–C(1)–H(1)	109.5
O(1)–C(3)–C(2)	122.81(13)	O(3)–C(6)–C(5)	123.00(12)	C(2)–C(1)–H(2)	109.5
O(1)–C(3)–C(4)	117.15(12)	O(3)–C(6)–C(7)	117.98(11)	C(2)–C(1)–H(3)	109.5
C(2)–C(3)–C(4)	120.04(14)	C(5)–C(6)–C(7)	119.01(13)	H(1)–C(6)–H(2)	109.5
O(2)–C(4)–C(3)	116.35(13)	O(4)–C(7)–C(2)	120.85(12)	H(1)–C(6)–H(3)	109.5
O(1)–C(4)–C(5)	121.40(12)	O(4)–C(7)–C(6)	115.71(13)	H(2)–C(6)–H(3)	109.5
C(3)–C(4)–C(5)	122.23(11)	C(2)–C(7)–C(6)	123.43(12)		

Table 2. Result of crystal toxicity.

Concentration (ppm)	Compounds	Total amount (Each group)	Survival amount					Mortality (%)
			Test group 1	Test group 2	Test group 3	Test group 4	Average	
10	Oosporein	10	0	0	0	0	0	100
	Podophyllotoxin	10	0	0	0	0	0	100
5	Oosporein	10	0	0	0	0	0	100
	Podophyllotoxin	10	1	0	0	0	0	100
3	Oosporein	10	2	3	3	2	3	70
	Podophyllotoxin	10	2	2	2	3	2	80
1	Oosporein	10	6	6	5	4	5	50
	Podophyllotoxin	10	5	5	5	6	5	50
Negative control	Distilled water	10	10	10	10	10	10	0

Table 3. Antitumor activity test of oosporein.

Compound	HL-60		A549	
	IC ₅₀ (µM)	95% Confidence interval	IC ₅₀ (µM)	95% Confidence interval
Oosporein	28.66	27.42 – 30.00	oosporein	28.66
VP-16	0.20	0.19 – 0.22	VP-16	0.20

IC₅₀ = Half maximum inhibitory concentration.

We speculated that the strain ZJWCF079 continued to produce active substances from 4 to 5 days and from 6 to 8 days, and may also absorb active substances for their metabolism to biosynthesize other metabolites, making the inhibition rate to drop temporarily.

When the crystal was obtained by chemical methods, its structure was determined by X-ray single crystal diffraction. Oosporein a widespread metabolite of soil-dwelling fungi

(Strasser et al., 2000), showed moderate antibiotic (Brewer, 1984; Strasser, 2000) and antifungal activities against *P. infestans* and some other phytopathogenic fungi (Toshinori et al., 2004). Oosporein, having being known for almost six decades, is a symmetrical red colored 2, 5-dihydroxybenzoquinone derivative biosynthesized by some soil borne fungi. The major secondary metabolite of the entomopathogenic fungi *Beauveria brongniartii* was

successfully used as a biological control agent against the European cockchafer *Melolontha melolontha* (Ulrich et al., 1950). In our study, oosporein had antifungal activity against *R. solani*, *B. cinerea* and *P. ultimum*, and is especially sensitive to *R. solani* and *P. ultimum*. Acute toxicity test of *A. salina* showed that oosporein also had great toxicity. That was why oosporein was used as biological control agent against the European cockchafer *M. melolontha* (Ulrich et al., 1950). Pronounced nephrotoxic activities of oosporein were found in feeding experiments using purified oosporein in artificial media (Cole et al., 1974). Although there are some confusion in the literature, and the data about nephrotoxicity of oosporein are not congruent (Astrid, 2004), no report about antitumor activity had been recorded. As shown in Table 3, oosporein showed some antitumor activity on HL-60 and A549. Though the effect of oosporein on cancer cell was lower than that of VP-16, the chemical and spatial structure was modified to improve activity. Besides, oosporein mixed with other anticancer drugs is suspected to be a more effective cure for cancer (Xiao et al., 2009).

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