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Antimicrobial activities of selected *Cyathus* species

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Abstract

Twelve selected *Cyathus* species were tested for their abilities to produce antimicrobial metabolites. Most of them were found to produce secondary exo-metabolites that could induce morphological abnormalities of rice pathogenic fungi *Pyricularia oryzae*. Some extracts from the cultivated liquid obviously inhibited human pathogenic fungi *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*. Activities against six human pathogenic bacteria were also obtained from some of these extracts.

Key words: active metabolites, Bird's nest fungi, morphological abnormalities

Introduction

As the name suggests, fruit bodies of Bird's nest fungi look like small bird's nests full of eggs. These eggs are small capsules known as peridioles that contain spores. They belong to the family Nidulariaceae with the most common genera been *Nidula*, *Cyathus*, and *Crucibulum*. They are found most often on decaying wood, small twigs, tree fern debris and sometimes on animal dung. *Cyathus* is a widely distributed genus all over the world [1]. In 1971, Albutt et al. [2] succeeded in growing *Cyathus helenae* in liquid medium and found antibiotic activities from the metabolites. Ayer & Ttaube [3] reported cyathin complex, a new class of diterpenoids, from *C. helenae* in 1973. Studies on the metabolites from *Cyathus* spp. have thrived for about 20 years since then. A number of new compounds were reported from various species, such as xanthone from *Cyathus intermedius* [4], striatin from *C. striatus* [5], cyafrin from *C. africanus* [6] and cyathatriol from *C. earlei* [7]. Subsequent studies reported many new substances from specific species. For example, cybullol, cybrodins and bullerone were reported from *C. bulleri* [8–10]. Some of them such as cyathins showed obvious activities against Gram-negative and Gram-positive bacteria, yeasts and moulds [2], and striatin against Nocadia [5]. Nevertheless, many species of *Cyathus* are still less documented for bioactive compounds. In this report we evaluate the potential bioactivities of 12 selected strains of *Cyathus* spp. against tested microoganisms.

Materials and methods

Production and extraction of metabolites

Twelve strains of *Cyathus* spp. were obtained from Prof. T.X. Zhou, Faculty of Conservation Biology, Southwest Forestry College of China. Two liquid media, Martin medium (1% glucose, 0.5% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 6.0) and potato dextrose broth (20% potato, 1.5% sucrose, pH 6.0) were used to cultivate these fungi. Slant tube cultures were inoculated into four flasks, each 500 ml Erlenmeyer flasks containing 150 ml of the above media. These flasks were cultivated on rotary shaker (180 rpm) for 15 days at 25 °C. Mycelia were removed by filtration through filter paper under reduced pressure and the clear culture broths were extracted twice with equal volume of ethyl acetate/chloroform/methanol (3:2:1, v/v/v). The resulted solutions were evaporated under vacuum. Each residue was dissolved in 2 ml of 10% methanol solution and stored at 4 °C.

Inducing morphological abnormalities of Pyricularia oryzae

Assays were carried out according to method described by Kobayashi [11]. Rice pathogenic fungus *P. oryzae* was inoculated onto PDA plate (20% potato, 1.5% sucrose, 2.0% agar) and incubated at 27 °C for 10 days. Conidial suspensions were obtained from the plates by washing off the conidia with sterilized water. Fifty microlitres conidial suspension (10^5 spores/ml) was mixed with 50 μl Sabouraud liquid medium (1.0% peptone, 0.5% NaCl, 4.0% glucose) and 50 μl crude extract solution in a 0.5 ml Eppendorf tube. The mixtures were incubated for 16 h at 27 °C. Resulted mixtures were observed under interference contrast microscope for the spores and mycelia morphological characters.

Antifungal activity assay

Antifungal activities against human pathogenic fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*) were tested by agar diffusion assay method. Conidial suspensions of tested organisms were prepared by washing off the conidia with sterilized water from Sabouraud dextrose agar plates which have been incubated at 27 °C for 7 days. Samples were prepared by pipeting 50 μl crude extract solution onto sterilized filter disks (Ø5 mm), which were then placed onto the Sabouraud dextrose agar plates homogenized with conidial suspension of the tested organisms. After 72 h, the diameters of the resultant zones of inhibition were measured.

Antibacterial activity assay

Antibacterial activities against six human pathogenic bacteria (*Escherichia coli*, *Mycobacterium tuberculosis*, *Staphylococcus albus*, *Streptococcus hemolyticus* α, *Streptococcus hemolyticus* β and *Streptococcus pneumonia*) were tested by agar diffusion assay method. Suspensions of tested organisms were obtained by incubating in meat broth (0.3% meat extract, 1% peptone, 0.5% NaCl, pH 7.4) with shaking for 2 days at 37 °C. Samples were prepared by pipeting 50μl crude extract solution onto sterilized filter disks (Ø5 mm), which were then placed onto the meat extract agar plates homogenized with suspensions of tested organisms. After 18 h, the diameters of resultant zones of inhibition were measured.

Thin layer chromatography of the crude extracts

TLC was carried out using Glass-backed TLC plates (silica gel 60GF254, 0.2 mm thick, Qindao Meijin Co. Ltd., China). Standard chromatograms of fungal extracts were prepared by applying 20 μl extract solution to a silica gel TLC plate and developed with chloroform/methanol (1:1; v/v) under saturated conditions. Chromatograms were detected by UV-light (254 and 365 nm), and by the colour reaction with 5% sulfuric acid-ethanol spraying solution after heating at 100 °C.

Results

Induced morphological abnormalities of Pyricularia oryzae

Morphological abnormalities of *P. oryzae* induced by crude extracts of *Cyathus* strains are shown in Table 1. Most of these extracts induced morphological abnormalities of *P. oryzae*. The morphological abnormalities including inhibition of conidial germination, spherical swelling, irregular swelling and abnormal branching are shown in Figure 1. Inducing abilities varied in different species or strains. *Cyathus intermedius* showed the strongest inducing ability, while *C. africanus* and *Cyathus* sp. 34 were the weakest. *Cyathus* sp. 37 didn't show activity. Media also have affections. Extracts from Martin media gave stronger activities than that ones from potato dextrose media.

Antifungal activities

The antifungal activities of the crude extracts of *Cyathus* strains are shown in Table 2. Most of extracts showed obvious activities against human pathogenic fungi *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*. These results were positively related to the abilities of *Cyathus* on inducing morphological abnormalities of *P. oryzae*.

Antibacterial activities

The antibacterial activities of *Cyathus* extracts are shown in Table 3. Among 12 *Cyathus* strains, *C. intermedius* is the most effective against tested bacteria, followed by *C. colensoi* and *C. pallidus*. The others showed very weak effect or no effect.

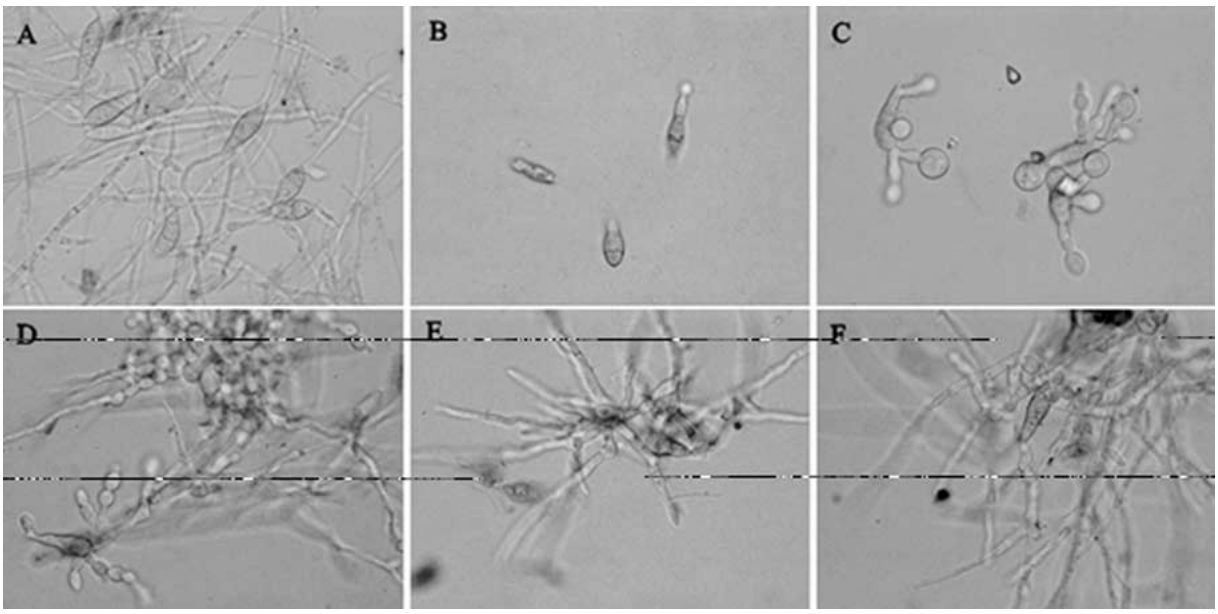

Figure 1. Morphological abnormalities of *Pyricularia oryzae* induced by crude extracts of selected *Cyathus* species. A. Natural characters of *Pyricularia oryzae*. B. Germination inhibition of *P. oryzae* induced by *Cyathus* intermedius; C. Morphological deformation of P. oryzae induced by C. colensoi; D. Morphological deformation of P. oryzae induced by C. gansuensis; E. Growth abnormalities of *P. oryzae* induced by *C. nigroalbus*; F. Growth abnormalities of *P. oryzae* induced by *C. africanus*.

Table 1. Morphological abnormalities of *Pyricularia oryzae* induced by crude extracts of selected *Cyathus* species

Selected *Cyathus* species	Extracts from Martin medium	potato dextrose broth
Cyathus olla	++	–
Cyathus africanus	+	–
Cyathus colensoi	++	++
Cyathus gansuensis	++	+
Cyathus sp. 39	++	–
Cyathus pallidus	++	++
Cyathus intermedius	+++	+++
Cyathus sp. 34	+	–
Cyathus sp. 37	–	–
Cyathus nigroalbus	+	++
Cyathus sp. 73	+++	++
Cyathus luxiensis	+++	+

+: Growth abnormality, general activity; ++: Spores could germinate and grow, but with serious morphological deformation, strong deformation activity; +++: Spores germination and growth were entirely inhibited, inhibited activity; –: Natural growth, no activity.

TLC of the crude extracts

Developed TLC plates with visualized spots are presented in Figure 2. There were intensive blue-white spots of all extracts under 365 nm UV light (Rf 0.75 $\sim$ 0.9). After colorized by 5% sulfuric acid-ethanol solution, polar spots appeared in several extracts (Rf $\sim$0.5).

Discussion

Numbers of potential antifungal antibiotics have been found by detection of their antifungal activities. Most of them however, failed in their action to discriminate fungal pathogens from the host due to high toxicity. Screening to detect activities of inducing morphological abnormalities of fungal cells will provide a chance to find selective antifungal agents [11–15]. For example, polyxin can cause formation of characteristic bulges or swellings on the growing hyphae and spores of *Pyricularia oryzae*. Such inhibitors block the synthesis of chitin in fungal cell wall and show very weak toxicity [12]. Under our experiments, most crude extracts of *Cyathus* strains showed abilities of inducing similar morphological deformation of *P. oryzae*. This indicates possibilities of the presence of compounds with selective activities from *Cyathus*. Subsequent results of antimicrobial activities have shown certain activities against tested organisms from crude extracts. While *Cyathus intermedius*, *C. gansuensis* and *C. luxi-*

Table 2. The antifungal activities of the crude extracts of selected *Cyathus* species

Selected *Cyathus* species	Antifungal activities of the extracts from									
	Martin medium					potato dextrose broth				
	C.n	C.a.	T.r	T.m	A.f	C.n	C.a	T.r	T.m	A.f
Cyathus olla	++	–	–	–	++	++	–	–	–	+
Cyathus africanus	++	+	–	–	+	–	–	–	–	+
Cyathus colensoi	+++	++	+	+	++	+++	+++	–	–	++
Cyathus gansuensis	+++	+++	+	–	++	++	++	+	–	++
Cyathus sp. 39	+++	–	–	–	++	–	–	–	–	++
Cyathus pallidus	++	–	–	–	+	++	++	–	–	++
Cyathus intermedius	+++	+++	++	–	+++	+++	+++	–	–	++
Cyathus sp. 34	++	++	–	–	+	–	+	–	–	–
Cyathus sp. 37	–	+	–	–	–	–	+	–	–	–
Cyathus nigroalbus	–	–	–	–	–	++	–	–	+	–
Cyathus sp. 73	+++	++	–	–	+	++	+	–	–	+
Cyathus luxiensis	+++	++	–	–	++	+++	+	–	–	++

Note. C.n: *Cryptococcus neoformans*; C.a: *Candida albicans*; T.r: *Trichophyton rubrum*; T.m: *Trichophyton mentagrophytes*; A.f: *Aspergillus fumigatus*.
+: Inhibited zone < 5 mm; ++: 5–15 mm; +++: > 15 mm; –: Absent.

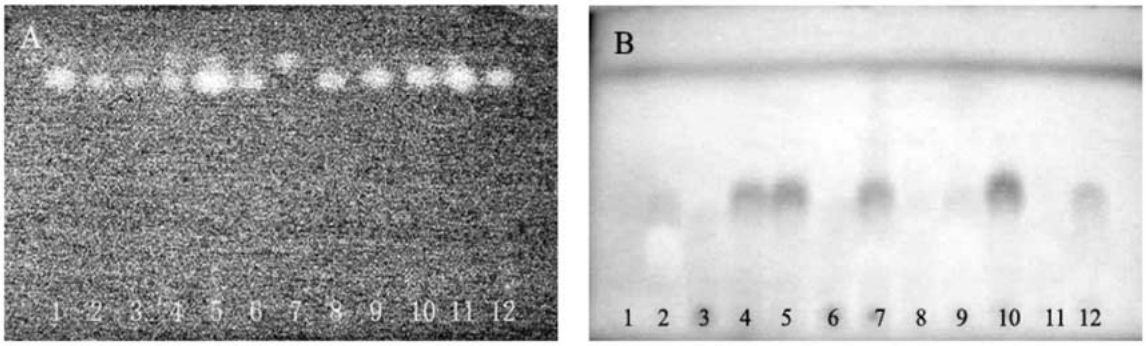

Figure 2. TLC separation of crude extracts of selected *Cyathus* species. Cultivation medium: Martin; Developing system: chloroform/methanol, 1:1, v/v; Detection: A: 365 nm UV light; B: 5% sulfuric acid-ethanol solution. Extracts: lane 1: *Cyathus olla*; lane 2: *C. africanus*; lane 3: *C. colensoi*; lane 4: *C. gansuensis*; lane 5: *Cyathus* sp. 39; lane 6: *C. pallidus*; lane 7: *C. intermedius*; lane 8: *Cyathus* sp. 34; lane 9: *Cyathus* sp. 37; lane 10: *C. nigroalbus*; lane 11: *Cyathus* sp. 73; lane 12: *C. luxiensis*.

ensis have strong activities of inducing deformation to *P. oryzae*, they also show abilities of inhibition to human pathogenic fungi and bacteria.

There are also correlations between variations of TLC and activities in different *Cyathus* strains. According to TLC detection, some differences were found in 12 strains extracts. All strains had intensive blue-white spots under 365 nm UV light (Rf 0.75). Only *Cyathus intermedius* had an intensive blue-white spot above other spots (Rf 0.85) and presented relatively strong inhibitive activity in antibacterial tests. *C. africanus*, *C. gansuensis*, *Cyathus* sp.39, *C. intermedius*, *C. nigroalbus* and *C. luxiensis* had same spots (Rf 0.5) colored by 5% sulfuric acid-ethanol solution. Other strains did not indicate this character. These spots did not show absolutely positive correlations with inducing activities, because both kinds of extracts had activities. But it does indicate the existence of some different active mechanisms. Chemical investigations are worthy to be done to identify the functional structures and their differences.

Our experiment results provide chances to find more antimicrobial metabolites from more *Cyathus* species. There should be some active metabolites principles in fungi from this genus. Continued research on them may help to increase our knowledge.

Table 3. The antibacterial activities of the crude extracts of selected *Cyathus* species

Selected *Cyathus* species	Extract from Martin medium active against					
	E.a	M.t	S.a	S.h-α	S.h-β	S.p
Cyathus olla	–	–	–	–	–	–
Cyathus africanus	–	–	–	–	–	–
Cyathus colensoi	–	++	+	++	++	++
Cyathus gansuensis	–	–	–	–	–	–
Cyathus sp. 39	–	–	–	–	–	–
Cyathus pallidus	–	++	–	++	+	++
Cyathus intermedius	+++	+++	+++	+++	+++	+++
Cyathus sp. 34	–	–	–	–	+	–
Cyathus sp. 37	–	+	–	–	+	–
Cyathus nigroalbus	–	–	–	–	–	–
Cyathus sp. 73	–	–	+	–	+	–
Cyathus luxiensis	–	–	+	–	+	–

Note. E.a: *Escherichia coli*; M.t: *Mycobacterium tuberculosis*; S.a: *Staphylococcus albus*; S.h-α: *Streptococcus hemolyticus* α; S.h-β: *Streptococcus hemolyticus* β; S.p: *Streptococcus pneumonia*.
+: Inhibited zone < 5 mm; ++: 5–15 mm; +++: > 15 mm; –: Absent.

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