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Antibacterial activity in extracts of *Cylindrocolea recurvifolia* (Cephaloziellaceae, Marchantiophyta) and *Pleurozia subinflata* (Pleuroziaceae, Marchantiophyta)

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Abstract – Disc diffusion assay was used to screen for antibacterial activity of aqueous, alcoholic and ether extracts of two liverworts with different habitats: *Cylindrocolea recurvifolia* (Cephaloziellaceae) on moist rocks in shaded forest, and *Pleurozia subinflata* (Pleuroziaceae) on trunks or branches in forest canopy. The ether extracts of *C. recurvifolia* were active against all seven selected bacterial species, alcoholic extracts possess antibacterial activity against six of the seven, but aqueous extracts exhibit very weak antibacterial activity only against one of the seven bacterial species. The ether and alcoholic extracts of *Pleurozia subinflata* demonstrated antimicrobial activity against six of the seven, but aqueous extracts exhibit weak antibacterial activity only against two of the seven bacterial species. Antibacterial activity of the ether extracts, expressed as MICs and MBCs, was compared with three reference antibiotic drugs. This is the first report of antibacterial activity tested on plants of Cephaloziellaceae and Pleuroziaceae. The genus *Cylindrocolea* is newly reported for Guangxi Province of China.

alcoholic extract / antibacterial activity / China / *Cylindrocolea recurvifolia* / ether extract / Guangxi / liverwort / MBC / MIC / oil body / *Pleurozia subinflata*

INTRODUCTION

Bryophytes are one of the important sources of antibiotics and biologically active, naturally occurring compounds (McCleary *et al.*, 1960; Zinmeister *et al.*, 1991). Their economic uses have been documented recently by Frahm (2004). Numerous recent studies also showed that bryophytes, particularly liverworts, contain a number of antibacterial compounds (Asakawa, 1998; Basile *et al.*, 1998a). Although China has a particularly rich liverwort flora, antibacterial activity of their extracts has not been screened extensively in mainland China (Zhu *et al.*, 2002) owing to difficulties in the collection of pure samples from the wild and lack of expertise in species identification.

Cylindrocolea R.M.Schust., a small mainly Gondwanalandic genus, contains *ca* 12 species (Schuster, 2002). The East Asiatic taxon, *C. recurvifolia*

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(Steph.) Inoue, is the most common member of *Cylindrocolea* in China and Japan and it usually forms large and luxuriant populations on very wet rocks along river banks, as shown in Iwatsuki (2001). The large quantity of wild populations of this species in Taiwan and Jiangxi of China has led to the finding of a new gymnomitrane-type sesquiterpenoid (Wu & Kao, 2002) and a natural ledol (Wu *et al.*, 1996, species reported as *Cephaloziella recurvifolia*). Thus, *Cylindrocolea recurvifolia* became the first species of Cephaloziellaceae chemically investigated. Although *C. recurvifolia* has been well studied taxonomically in Japan (Inoue, 1974), its distribution in China is still poorly known.

Pleurozia Dumort., the only representative of Pleuroziaceae, is a highly specialized genus with 11 species (Thiers, 1993). Its diagnostic characters, which include apical cell with only two cutting faces, smaller saccate dorsal lobe of the leaf with an aperture complex, and dimorphic perianths in sterile and fertile condition, are unique among liverworts. Owing to the large plant size, many unique morphological characters, and interesting habitats, *Pleurozia* has attracted the attention of a number of chemists to investigate the chemical nature of the genus. Flavonoids in 10 species (Mues *et al.*, 1991) and terpenoids in two species [*P. acinosa* (Mitt.) Trevis. and *P. gigantea* (Web.) Lindb.] have been reported (Wu & Asakawa, 1988; Asakawa, 2004). *Pleurozia subinflata* (Austin) Austin is known from China, Hawaii, Japan, Sri Lanka, Thailand, and Vietnam (Thiers, 1993). It was listed as one of endangered bryophytes in China (Chen, 1993; Wu *et al.*, 1997, as *P. giganteoides* (Horik.) Inoue).

During an expedition to northeast Guangxi Province in Sept. 2004, we found that *C. recurvifolia* and *P. subinflata* are not rare locally, forming large populations. The aim of our study is to investigate the possible inhibitory activity of *C. recurvifolia* and *P. subinflata* against bacterial species. The present paper not only reports the first discovery of *Cylindrocolea* in Guangxi, China, it also shows the antibacterial activity of cephaloziellaceous and pleuroziaceous plants for the first time.

MATERIAL AND METHODS

Liverwort collections

In September 2004, samples of *Cylindrocolea recurvifolia* were collected from a single sterile population growing on moist rocks at 550 m at Longtangjiang, Huajiang, Xinan Co., Guangxi Province. Collections of several fertile populations of *Pleurozia subinflata* were also made from forest canopy at 2000 m at Maoershan, Xinan Co., Guangxi, in September 2004. The voucher specimens, *R.-L. Zhu 20040908h* for *C. recurvifolia,* and *R.-L. Zhu 20040909-63* for *P. subinflata,* are deposited in the herbarium of East China Normal University (HSNU).

Testing microorganisms

Seven bacterial species were selected for the antibacterial tests. They are Gram positive (G+): *Bacillus megaterium, Bacillus subtilis, Bacillus thuringiensis, Staphylococcus aureus*, and Gram negative (G-): *Escherichia coli, Pseudomonas aeruginosa*, and *Pseudomonas putida*.

Extraction and initial screening

The liverwort materials were brought back to the laboratory in plastic bags. They were washed in distilled water, dried in an oven at 40 °C, and ground into powders. Ten grams of each liverwort species were separately shaken in 100 ml 95% ethanol, distilled water, and ether overnight. Extracts were then filtered and stored at 4 °C. For screening, the method of disc diffusion assay was used. The six mm sterilized filter paper discs that were soaked in the extracts overnight, were placed, after drying, onto LB agar plates grown with the testing bacteria. The inhibitory activity was indicated by a clear zone around the disc after incubation at 37 °C for 24 h, and the width of the clear inhibitory zone was measured. Cultures prepared in the same way but without liverworts were used as controls. Three antibiotic drugs, Amoxicillin, Cefradine, and Ciprofloxacin Hydrochloride, dissolved in distilled water (10 μg/ml) were used as references. The results of initial screening are shown in Tables 1 and 2.

Table 1. Antibacterial activities of *Cylindrocolea recurvifolia* shown in disc diffusion assays. The strength of the activity is represented by the width of inhibition zone expressed in mm.

Bacteria	*Width of inhibition zone (mm)*					
	Water extract	*Alcohol extract*	*Ether extract*	*Cefradine*	*Amoxicillin*	*Ciprofloxacin Hydrochloride*
Bacillus megaterium	0	0.9	0.4	9.0	7.9	7.4
B. subtilis	0.2	0.7	0.6	9.2	7.2	9.0
B. thuringiensis	0	0.5	0.8	8.0	6.8	9.0
Escherichia coli	0	0	0.6	10.0	9.5	10.4
Pseudomonas aeruginosa	0	0.5	0.6	5.2	5.0	6.9
P. putida	0	0.4	0.5	5.8	4.4	7.2
Staphylococcus aureus	0	0.4	0.2	10.3	9.4	10.0

Table 2. Antibacterial activities of *Pleurozia subinflata* shown in disc diffusion assays. The strength of the activity is represented by the width of inhibition zone expressed in mm.

Bacteria	*Width of inhibition zone (mm)*					
	Water extract	*Alcohol extract*	*Ether extract*	*Cefradine*	*Amoxicillin*	*Ciprofloxacin Hydrochloride*
Bacillus megaterium	0	0.3	0.5	9.0	7.9	7.4
B. subtilis	0	0.8	1.0	9.2	7.2	9.0
B. thuringiensis	0.2	0.8	0.8	8.0	6.8	9.0
Escherichia coli	0.1	0.3	0.5	10.0	9.5	10.4
Pseudomonas aeruginosa	0	0	0	5.2	5.0	6.9
P. putida	0	0.5	0.2	5.8	4.4	7.2
Staphylococcus aureus	0	0.5	0.5	10.3	9.4	10.0

Determination of MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration)

50g powders of liverwort samples were shaken overnight with 500 ml ether. Extracts were filtered, evaporated to a solid residue in a rotary evaporator. 100 mg of the dry residue were then diluted in 10 ml of sterile physiological Tris buffer (pH 7.4) solution. A serial 2-fold dilution of the plant extracts was made in sterile physiological Tris buffer (pH 7.4) and added to the LB agar plates to give a final concentration range of 4-1000 µg/ml. Each LB agar plate was incubated at 37 °C for 24 h. After incubation the lowest concentration of each extract showing no visible bacterial growth was recorded as MIC. Culture plates containing only sterile physiological Tris buffer (pH 7.4) were used as control.

The MBC determination was performed by transferring the extracts with concentrations higher than the MIC to fresh LB agar plates, following Basile *et al.* (1998b). The final concentrations of the extracts ranged from MIC to 2000 µg/ml. After incubation at 37 °C for 48 h, the MBC was reported as the lowest concentration of the extracts without visible bacterial growth. The MIC and MBC values were also determined for the three reference antibiotic drugs, Amoxicillin, Cefradine, and Ciprofloxacin Hydrochloride, using the aforementioned method. All the assays were carried out in triplicates. The MIC and MBC results are shown in Tables 3 and 4.

Table 3. Antibacterial activity of ether extract of *Cylindrocolea recurvifolia* and *Pleurozia subinflata* expressed as MIC (unit: µg/ml).

Bacteria	*C. recurvifolia*	*P. subinflata*	*Cefradine*	*Amoxicillin*	*Ciprofloxacin Hydrochloride*
Bacillus megaterium	500	125	4	16	8
B. subtilis	125	125	8	4	16
B. thuringiensis	125	125	8	8	4
Escherichia coli	250	250	8	4	0.25
Pseudomonas aeruginosa	500	500	32	16	32
P. putida	500	500	16	16	32
Staphylococcus aureus	500	250	2	0.5	1

Table 4. Antibacterial activity of ether extract of *Cylindrocolea recurvifolia* and *Pleurozia subinflata* expressed as MBC (unit: µg/ml).

Bacteria	*C. recurvifolia*	*P. subinflata*	*Cefradine*	*Amoxicillin*	*Ciprofloxacin Hydrochloride*
Bacillus megaterium	R_1	2000	32	R_2	64
B. subtilis	2000	2000	64	3	R_2
B. thuringiensis	1000	1000	64	64	64
Escherichia coli	2000	R_1	R_2	64	8
Pseudomonas aeruginosa	R_1	R_1	R_2	R_2	R_2
P. putida	R_1	R_1	R_2	R_2	R_2
Staphylococcus aureus	R_1	2000	32	8	16

R_1 = absence of inhibition at the highest concentration of extracts used (2000 µg/ml).
R_2 = absence of inhibition at the highest concentration of references used (64 µg/ml).

RESULTS AND DISCUSSION

The preliminary results of screening the liverwort extracts against the tested bacteria showed that alcoholic and ether extracts of *C. recurvifolia* and *P. subinflata* have detectable antibacterial activity. The aqueous extracts did not show recognizable activity on most bacteria, except for *B. subtilis* (Tab. 1), *B. thuringiensis* and *E. coli* (Tab. 2). According to Asakawa (1995), rich lipophilic terpenoids and aromatic compounds are the main biologically active secondary metabolites in liverworts, which are mainly present in oil bodies. These terpenoids do not easily dissolve in water, but can dissolve in alcohol and ether.

The alcoholic and ether extracts of *C. recurvifolia* and *P. subinflata* have apparent activities against both Gram-negative and Gram-positive bacteria. The ether extracts of *C. recurvifolia* were active against all seven selected bacterial species, and its alcoholic extracts demonstrated antimicrobial activity against six of the seven bacterial species. On the other hand, the ether and alcoholic extracts of *Pleurozia subinflata* possess antimicrobial activity against six of the seven bacterial species. However, all the responses were not satisfactory when compared with the antibacterial activities shown by the reference drugs used as controls, as shown in Tables 1 and 2. Such a result is similar to the conclusion drawn by So and Chan (2001) from their work done on some common liverworts collected from Hong Kong.

Since the initial screening already showed that the ether extracts exhibited broad-spectrum antibacterial activity, only ether extracts were used to determine MIC (Tab. 3) and MBC (Tab. 4) in the study. The MIC results showed that the ether extracts of *C. recurvifolia* exhibited a lower inhibitory activity against *S. aureus* and *B. megaterium* (Tab. 3), compared with *P. subinflata*. One of the reasons may be the lower contents of oil bodies in the leaf cells of *C. recurvifolia*, which are smaller and fewer than those in *P. subinflata*. In very fresh material of *C. recurvifolia* collected from Guangxi, oil bodies are almost homogeneous to very weakly granular, 2-4.2 × 1.5-2.6 mm, and about (2-) 3-4 (-6) per leaf cell. However, in *P. subinflata*, the oil bodies are of compound type, 2.4-5.6 × 1.6-3.2 mm, and about 4-12 per leaf cell, in fresh material obtained from Guangxi. As to the results of MBC measurement, the antibacterial activity of their ether extracts, however, did not show significant differences (Tab. 4), even though *P. subinflata* differs from *C. recurvifolia* in morphology and habitat, as mentioned above.

The broad spectrum of antibacterial activity shown in the present study suggests that *C. recurvifolia* and *P. subinflata* are two liverworts worthy of further investigation for the identification of their definitive antibacterial compounds and the nature of other potentially biologically active ingredients. We hope that more bryophytes will be screened in China for their natural antibacterial activities in order to help in the development of new and effective commercial drug products derived from bryophytes.

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