ORIGINAL ARTICLE

Antimicrobial activities of the crude methanol extract of *Acorus calamus* Linn.

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Abstract

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A partially-purified fraction obtained from column chromatographic preparation of the crude methanol extract of *Acorus calamus* Linn. rhizomes was investigated for its antimicrobial activities on various microorganisms including bacteria, yeasts and filamentous fungi. It exhibited high activity against filamentous fungi: *Trichophyton rubrum, Microsporum gypseum*, and *Penicillium marneffei* with IC_{50} values of 0.2, 0.2 and 0.4 mg/ml, respectively. However, it showed moderate activity against yeasts: *Candida albicans, Cryptococcus neoformans* and *Saccharomyces cerevisiae* (MIC 0.1-1 mg/ml) and low activity against bacteria (MIC 5->10 mg/ml). Scanning electron microscopic observation revealed that hyphae and conidia treated with this fraction were shrunken and collapsed, which might be due to cell fluid leakage.

Key words : *Acorus calamus*, antibacterial, antifungal, β- asarone fraction

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ฤทธิ์ต้านจุลินทรีย์ของสารสกัดเมธานอลจากว่านน้ำ (*Acorus calamus* Linn.)

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การทดสอบฤทธิ์ต้านจุลินทรีย์ของส่วนสกัดกึ่งบริสุทธิ์ ที่ได้จากการแยกสารสกัดหยาบเมธานอลของเหง้าว่านน้ำ ด้วย column chromatography ต่อเชื้อจุลินทรีย์ต่าง ๆ ได้แก่ แบคทีเรีย ยีสต์ และรา พบว่ามีฤทธิ์ต้านรา *Trichophyton rubrum, Microsporum gypseum* และ *Penicillium marneffei* ในระดับสูง โดยมีค่า IC_{50} เท่ากับ 0.2, 0.2 และ 0.4 มก./มล. ตามลำดับ มีฤทธิ์ต้านยีสต์ *Candida albicans, Cryptococcus neoformans* และ *Saccharomyces cerevisiae* ในระดับปานกลาง (MIC 0.1-1 มก./มล.) และมีฤทธิ์ต้านแบคทีเรียในระดับต่ำ (MIC 5->10 มก./มล.) เมื่อศึกษาด้วยกล้องจุลทรรศน์อิเลกตรอนชนิดส่องกราดพบว่าสารสกัดทำให้เส้นใยราและโคนิเดียมีความผิดปกติ หดตัวเหี่ยวย่น ทั้งนี้อาจเนื่องมาจากส่วนสกัดทำให้เกิดการรั่วไหลของของเหลวภายในเซลล์

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Acorus calamus Linn. (family Araceae) commonly known as "sweet flag" or Waan-Nam, is a well known medicinal plant. The rhizomes were utilized extensively by the Chinese, Indians and American Indians as well as by other cultures, and many of these uses continue to this day (Motley, 1994) including in Thai traditional medicine (Anonymous, 2000). The rhizomes are considered to possess anti-spasmodic, carminative and anthelmintic properties and also used for treatment of epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and tumors. It is listed as an insecticide, an antifungal agent, an antibacterial agent and a fish toxin (Anonymous, 1975). As part of a search for antimicrobial compounds from plants at the Natural Products Research Unit, Prince of Songkla University, Thailand, we found that one of partially-purified fraction obtained from the crude methanol extract of *A. calamus* rhizomes showed antimicrobial activity. Therefore, we report here the antibacterial and antifungal properties of this fraction which contained β–asarone as a major component according to proton nuclear magnetic resonance spectroscopy (^{1}H NMR).

Materials and Methods

Plant material and extraction

Rhizomes of *A. calamus* were collected from Songkhla Province, Thailand, in June 1997. The powder of dried rhizomes (1 kg) was marcerated in 8 l of analytical grade methanol at room temperature for 3 days, then filtered and evaporated to dryness in a rotary evaporator. The methanolic extract was further dissolved in ethyl acetate. The ethyl acetate soluble fraction was separated by quick column chromatography, eluting with a gradient system of increasing polarity (petroleum ether, dichloromethane, ethyl acetate and methanol). Nine fractions were subsequently collected and antimicrobial activity was screened. The third fraction was the most active fraction with the yield of 12.7% w/w. The major component of this fraction as confirmed by ^{1}H NMR is β-asarone.

Microorganisms and media

The microorganisms used in this study were clinical isolates of pathogenic bacteria (methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus* sp., *Escherichia coli* and *Pseudomonas*

aeruginosa), yeasts (*Cryptococcus neoformans* and *Candida albicans*) and filamentous fungi (*Microsporum gypseum, Trichophyton rubrum* and *Penicillium marneffei*). *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922 and *Saccharomyces cerevisiae* were used as standard strains.

Bacteria were grown on Mueller Hinton agar, MHA (Difco, USA) at 35ºC. Fungi were cultured and maintained on Sabouraud's dextrose agar, SDA (Difco, USA) at 25 and 35ºC.

Antibacterial assays

The disk diffusion method (Lorian, 1996) was used to screen the antibacterial activity of the third fraction (β-asarone). Sterile 6-mm diameter paper disks (Schleicher and Schuell, Germany) were impregnated with 10-mg (10 µl) of this fraction dissolved in 95% ethanol. Air-dried disks were placed on the inoculated MHA surface. Commercially available antibiotic disks of vancomycin (30 µg), tetracycline (30 µg), and gentamicin (10 µg) were used as standard antibiotics and disks impregnated with 10 µl of 95% ethanol as negative controls. Plates were incubated at 35ºC for 18 h and the inhibition zone diameter was measured. The tests were performed in duplicate.

The minimum inhibitory concentrations (MICs) were performed by the modified agar dilution method (Lorian, 1996). Serial 2-fold dilutions of the β-asarone were mixed with melted MHA in the ratio of 1:100 to give the final concentrations of 100-0.78 mg/ml in 9 cm diameter plates. Sterile membrane filter was laid on the surface of the agar. Inoculum suspension (2 µl) was inoculated on the membrane filter using multipoint inoculator (10^4 CFU/spot). Plates were incubated at 35ºC for 18 h. MICs were recorded by reading the lowest concentration that inhibited visible growth. The membrane filter was then transferred onto a new MHA plate and incubated at 35ºC for 18 h. Minimum bactericidal concentrations (MBCs) were recorded as the lowest concentrations that showed no growth on β-asarone fraction-free plates. Its effect on bacterial cells was also studied by scanning electron microscopy (SEM).

Antifungal assays

The antifungal assays against yeasts were performed as the antibacterial assays described above with the replacement of SDA as an assay medium and the minimum fungicidal concentrations (MFCs) were recorded. Amphotericin B (Bristol-Myers, Germany) was used as a positive control. Plates were incubated at 35ºC for 24 h (*C. albicans*) and 48 h (*C. neoformans* and *S. cerevisiae*).

Hyphal growth inhibition test was used to determine the antifungal activity. The procedure used in the hyphal growth inhibition test has been described previously (Picman *et al*., 1990). Briefly, dilutions of the test solutions dissolved in 95% ethanol were added to sterile melted SDA at 45ºC at the ratio of 1:100 to give final concentrations of 1, 0.8, 0.6, 0.5, 0.4, 0.2 and 0.1 mg/ml. The resultant solution was thoroughly mixed and approximately 100 µl was dropped into each sterile 1.5-cm diameter well-microscopic slides. Plugs of 1 mm of fungal mycelium cut from edge of active growing colony were inoculated in the center of the agar well and incubated in a humid chamber at 25ºC. Control cultures received an equivalent amount of 95% ethanol. Eight replicates were used for each concentration. Radial growth was measured when the control colonies almost reached the edge of the wells. Results were expressed as the percentage of hyphal growth inhibited (Gamliel *et al*.,1989). Concentration response curves were prepared in which the percentage of hyphal growth inhibition was plotted against concentration. The concentration required to give 50% inhibition of hyphal growth (IC_{50}) was calculated from the regression equation. Miconazole (Sigma) was used as a positive control. The effect of β-asarone fraction on fungal hyphae and conidia was also studied by SEM.

Results

The antimicrobial activities of the β-asarone fraction and control drugs are shown in Table 1.

Table 1. Antimicrobial activities of the β-asarone fraction.

Test Microorgamisms	β-asarone fraction		Control drug		
	IZ (mm)	MIC/MBC or MFC (mg/ml)	Drug	IZ (mm)	MIC/MBC or MFC (μg/ml)
Bacteria					
Staphylococcus aureus ATCC25923	9.2	5/>10	Vancomycin	16.5	1/1
Methicillin-resistant *S. aureus* (MRSA)	10.8	5/>10	Vancomycin	16.3	1/1
Enterococcus sp.	NZ	-	Vancomycin	18.9	1/4
Escherichia coli ATCC25922	6.8	10/>10	Gentamicin	20.1	0.5/0.5
Enteroinvasive *Escherichia coli*	6.8	10/>10	Gentamicin	20.1	0.5/0.5
Pseudomonas aeruginosa	NZ	-	Tetracycline	19.4	4/4
Yeasts					
Candida albicans	9.3	0.12/0.25	Amphotericin B	14.6	0.1/0.1
Cryptococcus neoformans	24.8	0.5/0.5	Amphotericin B	15.5	0.1/0.2
Saccharomyces cerevisiae	10.0	1/1	Amphotericin B	14.6	0.1/0.1
Filamentous fungi		**IC_{50} (mg/ml)**			**IC_{50} (μg/ml)**
Microsporum gypseum	ND	0.2	Miconazole	ND	1.4
Trichophyton rubrum	ND	0.2	Miconazole	ND	10.6
Penicillium marneffei	ND	0.4	Miconazole	ND	1.0

NZ = no zone, IZ = inhibition zone, ND = not determined
Disk content: the β-asarone fraction (10mg), amphotericin B (50μg), gentamicin (10μg), tetracycline (30μg), vancomycin (30μg)

This fraction exhibited low inhibitory activity against *S. aureus* and *E. coli* with narrow inhibition zones of 6.8-10.8 mm and MIC values of 5-10 mg/ml. It had no activity against *Enterococus* sp. and *P. aeruginosa*. It also produced narrow inhibition zones against *C. albicans* and *S. cerevisiae* (9.3-10 mm), but a larger zone (24.8 mm) was observed in C. *neoformans* (Figure 1). However, significant antifungal effects which expressed as MIC and MFC of the β-asarone fraction against the 3 strains of yeasts were presented at the concentrations of 0.1-1 mg/ml. Amphotericin B, which was the positive control drug for yeasts, exhibited strong antifungal activity with the MIC and MFC values of 0.1 and 0.1-0.2 μg/ml, respectively. This fraction exhibited considerable antifungal activity against filamentous fungi in dose dependent manner (Figure 2). The IC_{50} values on hyphal growth of *M. gypseum*, *T. rubrum* and *P. marneffei* were 0.2-0.4 mg/ml. Miconazole, which was the positive control, exhibited strong antifungal activity with the IC_{50} on hyphal growth of 1-10 μg/ml.

Microscopic observation on the effect of the β-asarone fraction on bacterial cells and fungal hyphae was performed by SEM study. No morphological change was observed in treated bacterial cells. In contrast, the β-asarone fraction caused drastic morphological changes in the test fungi. The treated hyphae and conidia were shrunken and collapsed (Figure 3).

Discussion

The results obtained from this study show that the β-asarone fraction has stronger antifungal activity than antibacterial activity. The extracts of *A. calamus* have been found to possess an antibacterial activity (Grosvenor *et al*. ,1995, MacGaw *et al*., 2002, Rani *et al*., 2003), although a lack of antibacterial activity has also been reported (De *et al*., 1999). β-asarone in *A. calamus* rhizomes was demonstrated to have antibacterial activity (MacGaw *et al*., 2002). However, β-asarone concentrations vary markedly among the oil from *A.*

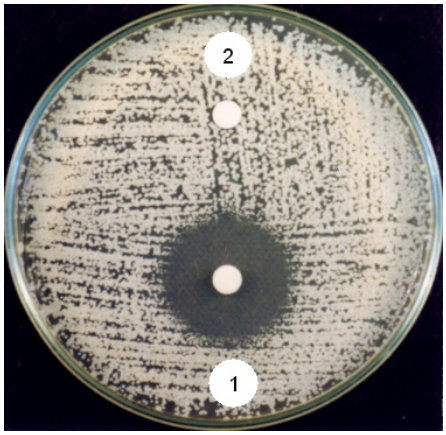

Figure 1. Disk diffusion assays of the β-asarone fraction (1) and 95% ethanol control (2) against clinical isolate of *Cryptococcus neoformans.*

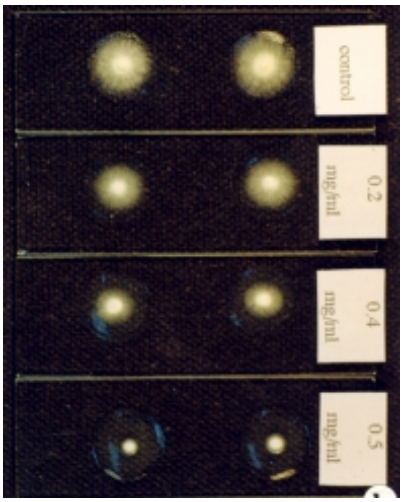

Figure 2. Hyphal growth inhibition assay of the β-asarone fraction against clinical isolate of *Microsporum gypseum* showing inhibition in dose-dependent manner.

calamus varieties. The tetraploid plant oil is high in β- asarone (90-96%). The triploid plants contain a small portion of β-asarone (5%) in their oil and the diploid plants lack β-asarone (Rost and Bos, 1979). *A. calamus* var. *americanus* comprises diploid members that are distributed from North America to Siberia. The triploid *A. calamus* var. *calamus* is distributed throughout Europe, temperate India and the Himalayan region, whereas the tetraploid one, *A. calamus* var. *angustatus* is found in eastern and tropical southern Asia (Rost, 1979). The investigated fraction is an antimicrobial-guided one of methanol extract of *A. calamus* containing β-asarone as a major component. In this study we found that the β-asarone fraction showed high antifungal activity against *M. gypseum, T. rubrum* and *P. marneffei* and had moderate activity against *C. albicans* and *C. neoformans*. Mungkornasawakul (2000) demonstrated the antifungal activity of crude dichloromethane extract of *A. calamus* rhizomes by TLC-bioassay using *Cladosporium cladosporioides* and cis-asarone was found to be the main compound. Thirach *et al*. (2003) reported that the ethanol extract of *A. calamus* inhibited clinical isolates of *C. albicans* and *C. neoformans* with the MIC/MFC values of 28.8/>75 and 3.02/30.8 mg/ml.The MIC/MFC values of the β-asarone fraction in our study was lower than those of Thirach *et al*. (2003) since it mainly contains β-asarone.

The effect of this fraction on cells of *S. aureus* studied by SEM showed no morphological change in the tested bacteria indicating that the active compound, β-asarone, had no effect on bacterial cell wall and membranes. In contrast, microscopic observation on the effect of this fraction on fungal hyphae and conidia showed drastic morphological alterations with shrunken and collapsed form. This could be due to the leak in the cell wall or perhaps some alteration in the membrane permeability.

Acknowledgements

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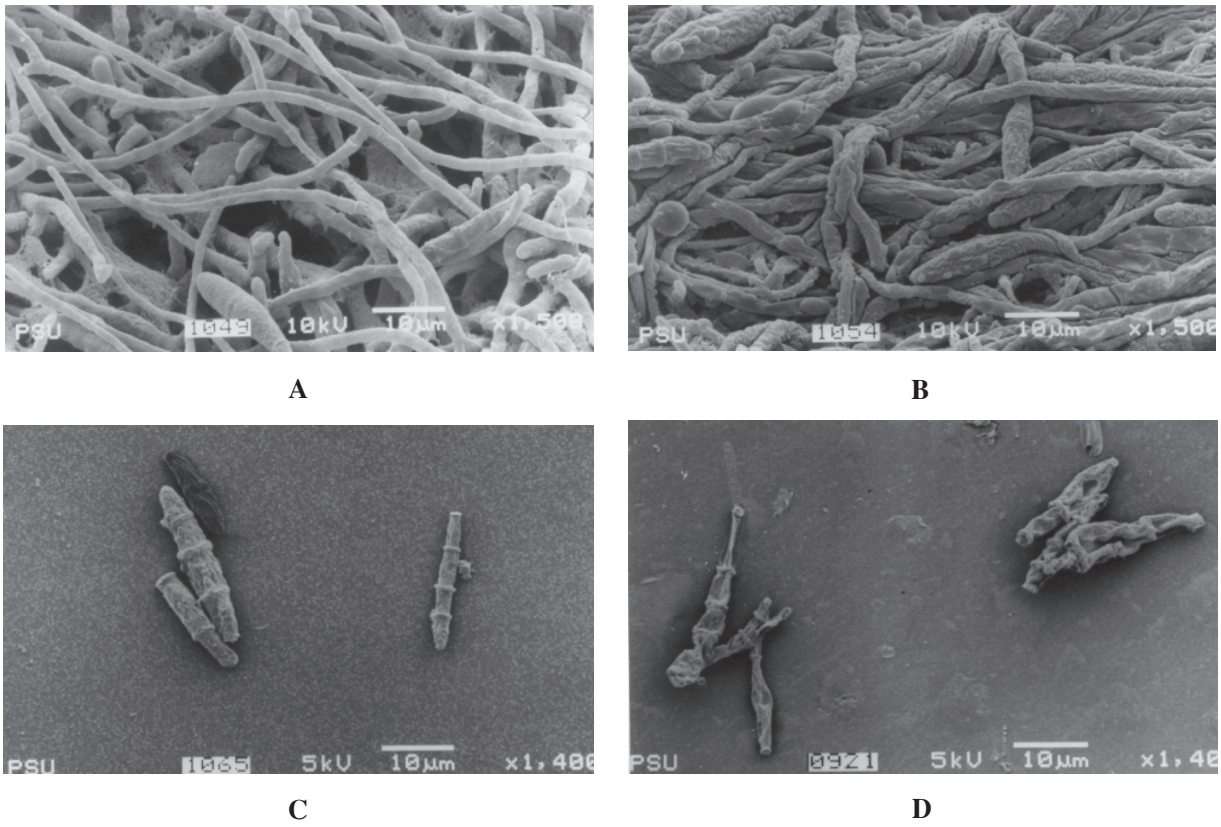

Figure 3. Scanning electron microscopy study of *Microsporum gyseum* after exposure to 100 mg/ml of the β-asarone fraction for 4 days. A and C. Control cultures which were exposed to 95% ethanol for 4 days, showing smooth walled hyphae and conidia (1,500 X). B and D. Treated cultures which were exposed to the extract showed collapsed and shrunken hyphae (1,500 X) and conidia (1,400 X).

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