SHORT COMMUNICATION

Antifungal activity from leaf extracts of *Cassia alata* L., *Cassia fistula* L. and *Cassia tora* L.

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Abstract

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Crude methanol extracts from leaves of *Cassia alata, Cassia fistula* and *Cassia tora* were investigated for their antifungal activities on three pathogenic fungi (*Microsporum gypseum, Trichophyton rubrum* and *Penicillium marneffei*). Among 3 species, *C. alata* was the most effective leaf extract against *T. rubrum* and *M. gypseum* with the 50% inhibition concentration (IC_{50}) of hyphal growth at 0.5 and 0.8 mg/ml, respectively, whereas the extract of *C. fistula* was the most potent inhibitor of *P. marneffei* with the IC_{50} of 0.9 mg/ml. In addition, it was found that all three *Cassia* leaf extracts also affected *M. gypseum* conidial germination. Microscopic observation revealed that the treated hyphae and macroconidia with leaf extracts were shrunken and collapsed, which might be due to cell fluid leakage.

Key words : *Cassia alata*, *Cassia fistula*, *Cassia tora*, antifungal

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ฤทธิ์ต้านราของสารสกัดจากใบชุมเห็ดเทศ (*Cassia alata* L.) ชัยพฤกษ์ (*Cassia fistula* L.) และชุมเห็ดไทย (*Cassia tora* L.)

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ทดสอบฤทธิ์ต้านราของสารสกัดหยาบเมธานอลจากใบชุมเห็ดเทศ ชัยพฤกษ์ และชุมเห็ดไทย ต่อเชื้อราก่อโรค 3 ชนิด (*Microsporum gypseum, Trichophyton rubrum* และ *Penicillium marneffei*) พบว่าสารสกัดจากใบชุมเห็ดเทศมีประสิทธิภาพดีที่สุดในการยับยั้ง *T. rubrum* และ *M. gypseum* โดยมีค่าความเข้มข้นที่ยับยั้งการเจริญของเส้นใยราได้ 50% (IC_{50}) เท่ากับ 0.5 และ 0.8 มก/มล ตามลำดับ ส่วนสารสกัดจากใบชัยพฤกษ์มีฤทธิ์ยับยั้ง *P. marneffei* ได้ดีที่สุด มีค่า IC_{50} เท่ากับ 0.9 มก/มล นอกจากนี้ยังพบว่าสารสกัดจากใบ *Cassia* ทั้ง 3 ชนิดมีผลต่อการงอกของ macroconidia ของ *M. gypseum* เมื่อศึกษาด้วยกล้องจุลทรรศน์อิเลกตรอนชนิดส่องกราดพบว่าสารสกัดทำให้เส้นใยราและ macroconidia มีลักษณะผิดปกติ หดตัวเหี่ยวย่น ทั้งนี้อาจเนื่องมาจากสารสกัดทำให้เกิดการรั่วไหลของของเหลวภายในเซลล์

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Due to the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effect of certain antimicrobial agents, there is a need to search for new agents. Many reports have shown that some *Cassia* species contain antimicrobial substances, particularly *Cassia alata* (Fuzellier *et al*., 1982; Caceres *et al*., 1991; Crockett *et al*., 1992; Caceres *et al*., 1993; Ibrahim and Osman, 1995; Agarkar and Jadge, 1999; Khan *et al*., 2001; Villasenor *et al*., 2002; Somchit *et al*., 2003). *Cassia* is a native plant in Southeast Asia, Africa, Northern Australia and Latin America (Parsons and Cuthbertson,1992). Many Cassia species are grown as ornamental plants throughout Thailand (Gritsanapan and Nualkaew, 2001). *C. alata, C. fistula,* and *C. tora* are recommended for primary health care in Thailand to treat ringworm and skin diseases (Farnsworth and Bunyaprapatsara, 1992). In this study we focused on the *in vitro* antifungal activity testing of methanol extracts of the leaves of these three *Cassia* species against clinical isolates of *Trichophyton rubrum, Microsporum gypseum* and *Penicillium marneffei*.

Materials and methods

1. Plant material and extraction

Leaves of *C. alata, C. fistula* and *C. tora* (Caesalpiniaceae) were collected from Songkhla Province, Thailand, during June to July, 1997. The specimens were compared with the voucher specimens at Prince of Songkla University Herbarium (*C. alata* No.185216, *C. fistula* No. 185224, *C. tora* No.185259). The leaves were oven- dried at 50ºC overnight. The ground leaves were macerated with methanol for 2 weeks at room temperature. This procedure was repeated twice. The filtrates were combined and evaporated under reduced pressure. The crude methanol extracts were dissolved in dimethylsulfoxide (DMSO) (Merck-Schuchardt, Germany). The yields of the methanol extracts were 6.4, 4.5 and 2.2%, respectively.

2. Fungi and media.

The pathogenic fungi used in this study were clinical isolates of *Microsporum gypseum, Trichophyton rubrum* and *Penicillium marneffei*

provided by Associate Prof. Dr. Angkana Chaiprasert, Mycology Laboratory, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University. Fungi were cultured and maintained on Sabouraud's dextrose agar, SDA (Difco, USA) at 25ºC.

3. Antifungal assays

Hyphal growth inhibition and conidial germination inhibition tests were used to determine the antifungal activity. The procedure used in the hyphal growth inhibition test has been described previously (Picman *et al*., 1990). Briefly, the test solutions dissolved in DMSO were added to sterile melted SDA at 45ºC at the ratio of 1:10 to give final concentrations of 1, 10 and 100 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 DMSO. 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). The active extracts that showed more than 50% hyphal growth inhibition at any concentration were further determined for the concentration required to give 50% inhibition of hyphal growth (IC_{50}). In this experiment, at least four concentrations of each active extract were tested against the test fungus. Concentration response curves were prepared in which the percentage of hyphal growth inhibition was plotted against concentration. From these, the IC_{50} was calculated from the regression equation. Miconazole (Sigma) was used as a positive control. The effect of the leaf extract on fungal hyphae was also studied by scanning electron microscopy (SEM).

The procedure for the conidial germination inhibition test was a modified method of Manandhar *et al*. (1995). *M. gypseum* conidia were washed from 14-day old cultures and adjusted to 5×10^5 conidia/ml. Ten µl of serial 2-fold dilutions of each test solution and 90 µl of the conidial suspension were mixed in duplicate circle-marked slides and incubated in a humid chamber at 25ºC for 18 h. Control conidia received an equal amount of DMSO. The number of conidia germinated was scored to calculate the percentage inhibition of conidial germination. The IC_{50} for inhibition of conidial germination was calculated from the concentration response curves. The effect of leaf extracts on macroconidia of *M. gypseum* was also studied by SEM.

Results

The results of the antifungal activity of methanol extracts of *C. alata, C. fistula* and *C. tora* are shown in Tables 1 and 2. The hyphal growth

Table 1. Effect of *Cassia* leaf extracts on hyphal growth of human pathogenic fungi

Plant extracts	% Hyphal growth inhibition at selected concentrations* (mg/ml)											
	T. rubrum				*M. gypseum*				*P. marneffei*			
	1	10	100	IC_{50}	1	10	100	IC_{50}	1	10	100	IC_{50}
C. alata	71.4	100	100	0.5	66.3	100	100	0.8	3.3	77.0	100	6.6
C. fistula	61.1	100	100	0.8	42.1	100	100	1.8	53.8	100	100	0.9
C. tora	40.1	100	100	1.2	42.2	100	100	1.8	46.2	100	100	1.8
Miconazole (µg/ml)	ND	ND	ND	1.4	ND	ND	ND	10.7	ND	ND	ND	1.0

ND, not determined
***Values are mean of 8 replicates.**

Table 2. Effect of *Cassia* leaf extracts on conidial germination and germ tube length of *Microsporum gypseum* at different concentrations (mg/ml)

Plant extract	% Conidial germination inhibition[a]			IC_{50} (mg/ml)
	0.1	1	10	
DMSO control	0	0	0	-
C. alata	61.5	100	100	0.1
C. fistula	ND	0	100	4.0
C. tora	ND	0	100	4.1
Miconazole (µg/ml)	0	ND	ND	0.04

ND, not determined
[a] , Values are mean of 300 conidia (germinated and ungerminated) performed in duplicates

of *T. rubrum, M. gypseum* and *P. marneffei* were inhibited by the three extracts in a concentration-dependent manner. *T. rubrum* and *M. gypseum* were completely inhibited by the extracts at a concentration of 10 mg/ml, and *P. marneffei* at a concentration of 100 mg/ml. *C. alata* was the most active plant extract against *T. rubrum* and *M. gypseum* with an IC_{50} values of 0.5 and 0.8 mg/ml, respectively. For *P. marneffei, C. fistula* was the most potent inhibitor with an IC_{50} of 0.9 mg/ml (Table 1). The *Cassia* extracts also affected *M. gypseum* conidial germination as shown in Table 2. At the concentration of 10 mg/ml all extracts inhibited conidial germination. The *C. alata* extract showed the highest activity with the IC_{50} of 0.1 mg/ml. Microscopic observation on the effect of the leaf extracts on hyphae and macroconidia was performed by SEM study. All three extracts caused morphological changes in the test fungi. The most prominent change was seen in the treatment with *C. alata*. The treated hyphae of the three test fungi as well as the macroconidia of *M. gypseum* were shrunken and collapsed (Figures 1 and 2). Miconazole, which was the positive control, exhibited strong antifungal activity with the IC_{50} on hyphal growth and on conidial germination of 1- 10 µg/ml and 0.04 µg/ml, respectively.

Discussion

The results obtained from this study show that all three *Cassia* leaf extracts are inhibitory towards pathogenic fungi (*T. rubrum, M. gypseum* and *P. marneffei*). These findings confirm the traditional therapeutic claims for these herbs to treat ringworm and skin diseases. *P. marneffei* infections are common AIDS defining opportunistic infections among persons with HIV infections in northern Thailand (Chariyalertsak *et al*., 1996). These three *Cassia* species are excellent candidates for treatment of penicilliosis marneffei.

Previous reports of antimicrobial activity against human pathogens have been widely carried out for *C. alata* (Fuzellier *et al*., 1982; Palanichamy and Nagarajan, 1990; Crockett *et al*., 1992; Grosvenor *et al*., 1995; Ibrahim and Osman, 1995; Hofilena *et al*., 2000; Khan *et al*., 2001; Villasenor *et al*., 2002; Somchit *et al*., 2003). Fuzellier *et al*. (1982) found that 5% aqueous extract from leaves of *C. alata* and some of its components, rhein, emodol, 4,5-dihydroxy-1-hydroxymethylanthrone and 4,5-dihydroxy-2-hydroxymethylanthraquinone, had antifungal activity against some dermatophytes and yeast. Palanichamy and Nagarajan (1990) extracted *C. alata* leaves with petroleum ether followed by hot 85% ethanol under reflux and tested for its antifungal activity against *Candida albicans, Aspergillus fumigatus, Aspergillus flavus, Mucor* spp., *Rhizopus* spp. and dermatophytes; *Trichophyton mentagrophytes, T. rubrum* and *M. gypseum*. They reported that 20% w/v crude extract did not show any significant activity against the contaminant fungi, whereas 2.5 and 3% crude extract com-

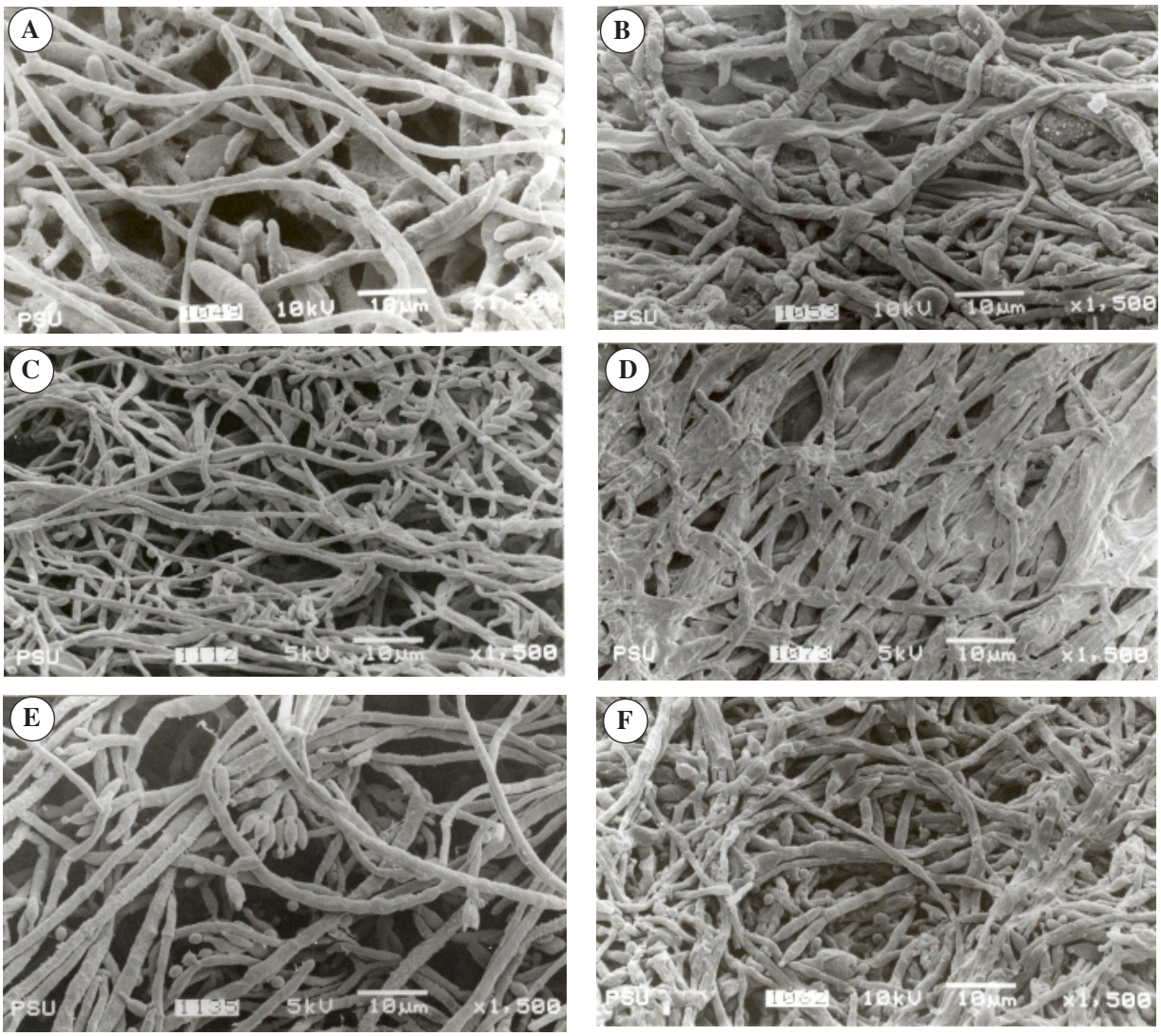

Figure 1. Scanning electron microscopy study of *Microsporum gyseum* (A and B), *Trichophyton rubrum* (C and D) and *Penicillium marneffei* (E and F) after exposure to 100 mg/ml *Cassia alata* leaf extract for 4 days. A, C and E. Control cultures which were exposed to DMSO for 4 days, showing smooth walled hyphae and conidia (1,500 X). B, D and F. Treated cultures which were exposed to the extract, showing collapsed and shrunken hyphae and conidia (1,500 X).

pletely inhibited the growth of dermatophytes. Most of the extracts tested, either extracted by aqueous, alcohol, hexane, petroleum ether or ethyl acetate exhibited anti- dermatophytic activity. In this study only methanol was used as the solvent; while Caceres *et al*. (1993) reported that ethanol was the best solvent. Only a few antibacterial investigations have been done for *C. fistula* (Patel and Patel, 1956; Samy *et al*., 1998; Ali *et al*., 1999). For *C. tora*, Acharya and Chaterjee (1975) reported that the major antifungal component extracted from defatted seed was chrysophanic acid-9-anthrone which was active against dermatophytes. Our findings confirm antifungal

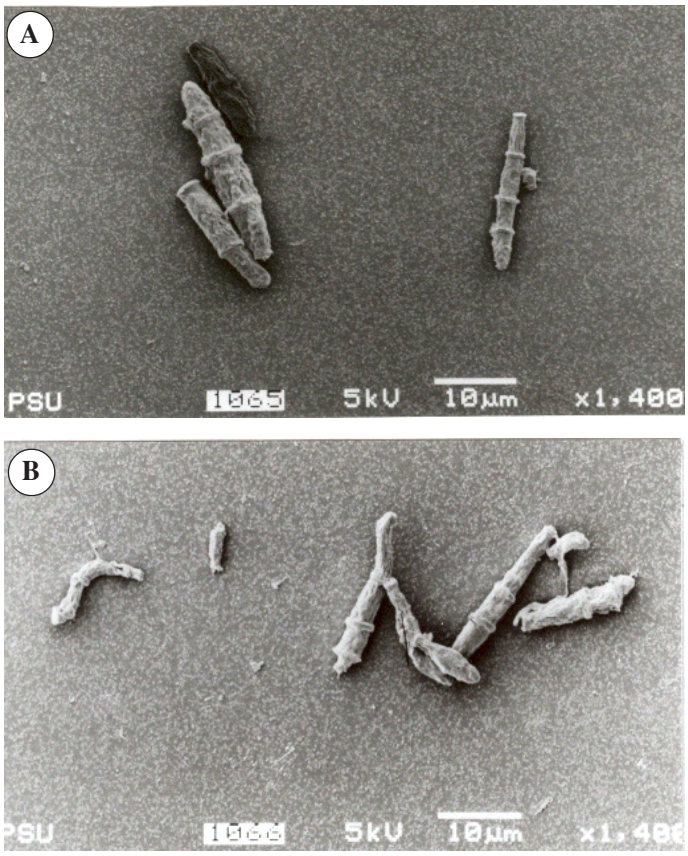

Figure 2. Scanning electron microscopy study of *Microsporum gyseum* macroconidia after exposure to 0.1 mg/ml *Cassia alata* leaf extract for 18 h. A. Control macroconidia which were exposed to DMSO for 18 h, showing normal shaped macroconidia (1,400 X). B. Treated macroconidia which were exposed to the extract, showing abnormal macroconidia (1,400 X).

activity of these three *Cassia* leaf extracts. Among the 3 species of *Cassia, C. alata* was the most active plant against *T. rubrum* and *M. gypseum* and *C. fistula* was against *P. marneffei*. The effect of extracts on macroconidia of *M. gypseum* was also examined. It was found that the IC_{50} values for the inhibition of hyphal growth were lower than those for the inhibition of conidial germination, except for *C. alata* and miconazole. However, microscopic observation on the effect of the extracts on fungal hyphae and macroconidia showed similar morphological alteration with shrunken and collapsed form. This finding agrees with the previous report of Ibrahim and Osman (1995) on the effect of *C. alata* on macroconidia of *M. gypseum*. They explained that this phenomenon could be due to the leaks in the cell wall or perhaps some alteration in the membrane permeability, resulting in the loss of the cytoplasm. Agarkar and Jadge (1999) reviewed 25 references and found that about 26 species of genus *Cassia* have been reported to contain anthracene derivatives. According to Ibrahim and Osman (1995) chrysophanic acid and chrysophanol in the leaves of *C. alata* were claimed to have antifungal properties. Crude anthraquinones, rhein and aloe- emodine also showed antifungal activity against dermatophytes (Palanichamy and Nagarajan, 1990; Hofilena *et al.*, 2000). In *C. fistula*, flavone glycoside from seeds showed antimicrobial activity (Yadava and Verma,

2003) and in *C. tora*, chrysophanic- 9 anthrone (Acharya and Chaterjee, 1975; Hatano *et al*., 1999). The antifungal substances contained in the leaf extracts of these *Cassia* species may have similar mechanism that could be related to fluid leaks in cells. For follow-up research, it is important to determine the active components in each extract and confirm their mechanism.

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