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Full Length Research Paper

Screening of phytochemical from ethnomedicinal plants in Malaysia for use against toxigenic *Aspergillus flavus*

Yazdani, D.[1, 2], Zainal Abidin, M. A.[2]*, Tan, Y. H.[2], Kamaruzaman, S.[2] and Jaganath, I. B.[3]

[1]Department of Pharmacognosy and Pharmaceutic, Institute of Medicinal Plants, ACECR, Tehran, Iran.
[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[3]Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia.

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Medicinal plants have been used as traditional treatments for numerous diseases for thousands of years. Antifungal activity of phytochemicals extracted from nine plant species in Malaysia traditionally used for treatment of skin infections were tested against growth of aflatoxin-producing *Aspergillus flavus* isolated from rice grains. The results, evaluated by measuring the diameter of inhibitory zone in disk diffusion test, minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC), showed that chloroform soluble fractions of methanolic extract of *Alpinia galanga* (lengkuas) rhizomes could strongly inhibit the mycelia growth of *A. flavus*. The constituent of this phytochemical fraction was identified by HPLC-MS^2 using ESI ionization technique. In total, 10 compounds were identified based on MS^2 data and λ_{max} at 280 nm. These results suggested the potential of *A. galanga* for used as botanical fungicide.

Key words: *Alpinia galanga*, antifungal, *Aspergillus flavus*, traditional medicine, phytochemicals, LC-MS^2.

INTRODUCTION

Food contamination by mycotoxins is an important food safety concern for grains and other agricultural products. Food contaminated with mycotoxins, particularly aflatoxins, can cause sometimes fatal acute illness and are associated with increased cancer risks (Buzby, 2003).

In order to identify and develop novel antifungal agents, several plant extracts and compounds isolated were examined for their bioactivities. Previous researches on antifungal activity of extracts of several higher plants have indicated the possibility of exploiting natural antifungal agents for control of fungi although little are known in this field (Wannissorn et al., 2005).

Most of the secondary plant compounds utilized in modern medicines were first discovered through ethnobotanical investigations. Several studies have demonstrated demonstrated significantly higher rate of pharmacological activity in plant extracts used ethnomedically compared to extracts from randomly collected plants (Gurib-Fakim, 2006; Kothari, 2007). Carlson (2002) reported that 1.6% of the randomly collected plants were active compared to 15% of the plants used by the traditional Mayan healer. Over 15,000 flowering plant species were estimated in Malaysia's rainforests (Shippamann et al., 2002). In this study 9 plant species previously used as traditional medicines for treatment of fungi infections in Malaysia were screened against an aflatoxin-producing strain of *A. flavus* isolated from rice (Yazdani et al., 2010).

MATERIALS AND METHODS

Plant materials and extract preparation

9 plant species were selected based on previous ethnomedicinal

*Corresponding author. E-mail: zainal @ agri.upm.edu.my. Tel: + 603- 8946- 6986. Fax: + 603- 8656- 0698.

Table 1. Selected ethnomedicinal plant species for treatment of skin infections in Malaysia.

Plant scientific name	Part used	Family	References	Voucher specimen[a]
Alpinia galanga (L.)Wild	Rhizomes	Zingiberaceae	[b]	SK 1594/09
Alpinia conchigera Griff.	Rhizomes	Zingiberaceae	[c]	SK 1714/09
Artocarpus heterophyllus Lam.	Leaves	Moraceae	[d]	SK 1595/09
Capsicum frutescens L.	Leaves	Solanaceae	[d]	SK 1596/09
Euphorbia hirta L. Syn.*Chamaesyce hirta* (L.) Mill.	Shoots	Euphorbiaceae	[d]	SK 1597/09
Morinda citrifolia L.	Leaves	Rubiaceae	[e]	SK 1598/09
Piper betle L.	Leaves	Piperaceae	[d]	SK 1599/09
Pseuderanthemum crenulatum Radlk	Leaves	Acanthaceae	[e]	SK 1560/09
Stachytarpheta indica L. (Vahl.)	Leaves	Verbenaceae	[d]	SK 1561/09

a: Voucher specimens deposited in the Biodiversity Unit Herbarium, Institute of Bioscience, University Putra Malaysia; b: Ong and Norzalina (1999); c: Faridah and Nurulhuda (1999); d: Fasihuddin and Holdsworth (2003); e: Lin (2005).

ethnomedicinal studies, the selected plant species were collected from Universiti Putra Malaysia (UPM) campus and voucher specimens were deposited in the Biodiversity Unit's Herbarium, Institute of Bioscience, UPM (Table 1).

The fresh plant materials were washed and rinsed in tap water, dried at 40°C using forced air convention oven dryer and powdered by grinder machine (Cross Beater Mill SK 100, Germany). Powdered plant material (125 g) was extracted with methanol (1:6 w/v) by maceration for 72 h and filtered through Whatman No. 1 filter paper. The filtered fluid was evaporated to dryness under reduced pressure on a Buchi rotary evaporator model R-215 (Switzerland) to provide crude extract. Aliquots of crude extract which showed high inhibitory effect against fungus growth were suspended in methanol (90%) and sequentially extracted with an equal volume of hexane and chloroform to afford the fractions of hexane, chloroform and water (Jones and Kinghorn, 2006; Sarker et al., 2006). All fractions were concentrated in a rotary evaporator followed by a Freezone 6 freeze dryer (Labconco, USA). Dried extract was weighed and resuspended in 2% dimethyl sulfoxide (DMSO) in distilled water for preparation in appropriate concentrations. Benlate (50% WP) and amphotericin B (Cipla, India) used as controls.

Fungus and inocula preparation

The fungus strain used in this study was *A. flavus* UPMC 89 (NCBI accession no. GU076485), a strong aflatoxin-producing strain, obtained from Microbial Culture Collection Unit (UNiCC), UPM. The cultures were maintained on Czapek Dox agar (CDA) slants at 4°C. The fungus was grown on Czapek yeast agar (CYA: CDA+ 0.5% Yeast) at 28°C for 7 days. Spore suspension was prepared as described by Esteban et al. (2004) with few modifications. The fungus was grown on CYA at 28°C for 7 days. The conidia were harvested aseptically using 5 mL of sterile aqueous solution of tween 20 (0.5%, v/v). The conidia suspensions were filtered through sterile fine texture cloth and adjusted to a concentration of 2-3 × 10^6 spores/mL in sterile distilled water, corresponding to 0.15 to 0.17 absorption when a Varian Cary Model 50 spectrophotometer set at 530 nm was used.

Antifungal activity assay

Disk diffusion test

All fractions of crude extracts were preliminary tested for antifungal activity using disk diffusion method (Engelmeier and Hadacek, 2006). 100 µl of fungus spore suspension (3×10^6 spores/ml) were added to solid CYA medium in 9 cm plates and distributed uniformly using a sterile glass rod aseptically. The fractions were dissolved in 5% dimethyl-sulfoxide (DMSO) in water (v/v) to a final concentration of 10, 5, 2.5 and 1 mg/ml. Sterile paper discs (6 mm, Whatman, UK) were impregnated with 20 µl of each fractions while the control disks were impregnated with sterile distilled water and placed on the inoculated plates.The plates were incubated at 28°C and diameter of the inhibition zone (mm) around the disk was measured after 48 h, the experiment were replicated 3 times.

Determination of MIC and MFC

The fraction which exhibited the strongest inhibitory effect in disk diffusion test was tested for its MIC and MFC using the macro dilution technique described by Pujol et al. (1996) and Sanchez et al. (2005) with modifications. 200 µl of a fungus spore suspension (2-3×10^6 /ml) was added in test tubes at various concentrations (500, 250, 100, 50 µg/ml) of fraction in potato dextrose broth (PDB). The antifungal agent Benlate (50% WP) and amphotericin B (Cipla, India) were included in the assay as positive controls. Extraction free PDB was also used as a blank control. The tubes were incubated at 28°C for 2 days and fungal growth was observed visually throughout the incubation period. All experiments were replicated 4 times. The MIC was defined as the lowest concentration which inhibited the visible growth of fungus after incubation period. The MFC was determined by removing 10 µl of the contents from all tubes showing no visible growth and from the last tube to show growth (Pfaller, 2005).The samples were spotted onto CYA plates and incubated at 28°C for 3-7 days. The MFC was defined as the lowest concentration that did not allow the growth of colonies.

LC-MS^2 analysis of chloroform soluble fractions from *A. galanga*

For HPLC/UV/MS analyses, the chloroform-soluble fraction of A. galanga, was filtered through Whatman syringe filters (GD/x 13 mm, USA). The extracts were analyzed with a HPLC system consisting of an Agilent (1200 series, Germany) system with a diode array detector set at 280 nm. The injection (20 µl) was performed by an autosampler with an injection needle. The analytical column was a Thermo Hypersil Gold C18 (5 µm, 150 × 4.6 mm i.d, Thermo Scientific, Australia). Analytical separation was achieved at a flow

Table 2. Inhibitory effect of methanolic extracts of selected medicinal plants against *A. flavus.*

Plant species	Part used	Crude extract ratio (%w/w) [a]	Inhibition zone (mm); numbers in brackets are standard deviation [b]			
			10 mg/ml	5 mg/ml	2.5 mg/ml	1 mg/ml
A. galanga	Rhizomes	15	19 (1.15)	15 (1.4)	13 (0.8)	11 (0.9)
A. conchigera	Rhizomes	19	14 (0.5)	10 (0.5)	7 (0.5)	N
A. heterophyllus	Leaves	10	N	N	N	N
C. frutescens	Leaves	13	N	N	N	N
E. hirta	Shoots	14	N	N	N	N
M. citrifolia	Leaves	17	N	N	N	N
P. betle	Leaves	22	9.5(0.5)	N	N	N
P. crenulatum	Leaves	12	N	N	N	N
S. indica	Shoots	29	N	N	N	N
Control[c]	-	-	ND	ND	ND	11(1)

a. Crude extract/dried part used; b. average of 4 replications; c. Fungicide (Banlate) incorporated in disk diffusion test. N= No inhibition, ND=Not determined.

Table 3. Inhibitory effect of different fractions from *A. galanga* crude extracts against *A. flavus*.

Fraction	Inhibition zone (mm), numbers in brackets are standard deviation[a]			
	500 µg/ml	250 µg/ml	100µg/ml	50 µg/ml
Chloroform	13 (1)	11(0.5)	10 (0.9)	N
Hexane	9 (1)	N	N	N
Water	N	N	N	N
Control [b]	13(1)	13(1)	13(1)	13(1)

a. Average of 4 replicate; N= no inhibition; b. Fungicide (Banlate) incorporated at 1 mg/ml.

rate of 1 ml/min with the following gradient program: 4 min 95% A, 5% B; 30 min 60% A, 40% B; 38 min 5% A, 95% B; 39 min 95% A, 5% B. Eluent A consisted of water containing 0.5% (v/v) formic acid. Eluent B was acetonitrile containing 0.5% (v/v) formic acid. The temperature of column oven was set to 20°C. A 3200 Q-TRAP mass spectrometer (Applied Biosystems, USA) was connected to the LC via an electrospray ion (ESI) source. The spectrometer was operated in negative mode and the detected mass range was set to 100 to 800 m/z.

Table 4. MIC and MFC of chloroform fraction from *A. galanga* extract against *A. flavus*.

Fraction	MIC(µg/ml)	MFC(µg/ml)
A. galanga	25	50
Benlate (Control)	100	500
Amphotericin B (Control)	1	2.5

RESULTS AND DISCUSSION

Inhibitory effect of extracts against *A. flavus* using disk diffusion technique

Crude extract of *A. galanga* and A. conchigera exhibited inhibitory effects on mycelial growth of A. flavus. The former showed more activity with inhibition zone of 11 mm at concentration of 1 mg/ml.

None of the other tested crude extracts had presented inhibitory effect against *A. flavus* at concentrations of 1 to 5 mg/ml (Table 2). The crude extracts which showed no effect against *A. flavus* in concentration 1 mg/ml were ignored and only fractions of *A. galanga* crude extract were tested.

Within chloroform, hexane and aqueous fractions, the chloroform fraction showed the highest inhibitory effect with inhibition zone of 10 mm at concentration of 100 µ/ml (Table 3). Aqueous and hexane fractions did not show any inhibitory effect (Table 3).

MIC and MFC of *A. galanga*

Since the chloroform soluble fractions of the methanolic extract exhibited the strongest inhibitory effect on *A. flavus* (Table 3), the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) was performed only for this fraction. The chloroform fraction exhibited the lowest MIC and MFC values against *A. flavus* growth at 25 and 50 µg/ml respectively (Table 4). The MIC and MFC for controls fungicide (Benlate and

Table 5. Identified compounds in chloroform fraction of *A. galanga* using LC-MS^2 at 280 nm.

Peak	RT (min)	Compound	$[M-H]^-$ (m/z)	MS fragment ions(m/z)
1	14.30	Derivative of feruloyl tartaric acid	519	475, 457, 343, 325, 193, 149, 131
2	16.70	Coumaric acid	163	119, 117, 108
3	18.60	Cinnamic acid	147	129, 119,117,116, 103
4	22.11	Derivative of hepatanone	723	677, 659, 593, 451, 367, 341, 324, 309, 225
5	22.80	Diethylglucosyl hydroxycinnemate	499	455, 411, 367, 325, 249, 235, 205, 193, 147, 33,121
6	23.90	Glucosyl hydroxycinnamate	443	411, 381, 325, 309, 293, 223, 193, 163, 131
7	25.40	Caffeic acid glucoside	341	297, 270, 159, 161, 145, 135, 123, 109
8	26.46	Derivative of ferullic acid	371	205, 191, 176, 165, 161, 159, 148, 134, 119
9	27.60	Gingerol	313	295, 261, 247, 221, 193
10	33.11	Derivative of coumaryl acetate galactoside	775	551, 371, 205, 191, 175, 134

RT: retention time; $[M-H]^-$: negatively charged molecular ions

Amphotericin B) were 100 and 500 µg/ml and 1 and 2.5 µg/ml respectively against fungus tested (Table 4).

Alpinia galanga (Zingiberaceae) is widely cultivated in South and Southeast Asian countries. It robust is a, perennial and aromatic herb (Willdenow, 1797).The rhizome of this plant is a commonly used spice or ginger substitute for flavoring foods, and also in traditional medicines for several purposes, such as carminative, antiflatulent, antifungal, and anti-itching agents (Kaur et al., 2010). In Malaysian traditional medicine, the rhizomes are used for the treatment of skin infected with fungi (Ong and Norzalina, 1999). According to Khattak (2005), crude extract of *A. galanga* rhizome showed a moderate inhibitory effect (30%) against *A. flavus*.

HPLC-Tandem mass spectrometry (LC-MS^2) of extracted compounds from *A. galanga*

In total 10 compounds were identified in the chloroform soluble fractions of methanolic extract from *A. galanga* based on MS^2 data and λ_{max} at 280 nm. The results obtained from the LC-MS^2 are presented in Table 5.

Cinnamic acid and related aromatic fatty acids such as coumaric acid, caffeic acid and ferulic acid, are found in many plants. These unsaturated carboxylic acids are natural precursors of structurally related flavonoids. They constitute a large family of organic acids that have antibacterial, antifungal and antiparasitic activities (Narasimhan et al., 2004). In accordance with Barik et al. (1987) and Pancharoen et al. (2000), the major compounds isolated from *A. galanga* were cinnamic acid (RT: 18.6, m/z 147) and its derivatives consisting coumaric acid (RT: 16.7, m/z 163), diethylglucosyl hydroxycinnemate (RT:22.7, m/z 499), glucosyl hydroxycinnamate (RT: 23.9, m/z 443) and ferulic acid (RT: 26.4, m/z 371). Gingerol (RT:27.6, m/z 313), which was identified in the present study had been isolated from genus *Amomum* spp. (Zingiberaceae) by Pancharoen et al. (2000). However, it was not previously reported from genus *Alpinia*.

Conclusion

Higher plants have been described as chemical factories that are capable of synthesizing unlimited numbers of highly complex substances (Farnsworth, 1988). Several studies have demonstrated significantly higher rate of pharmacological activity in plant extracts used ethnomedically compared to extracts from randomly collected plants (Gurib-Fakim, 2006; Kothari, 2007). In accordance with Rios and Recio (2005) we ignored crude extracts with inhibitory effect of less than 1 mg/ml. Only *A. galanga* crude extract showed significant inhibitory effect against fungus at concentration of 1 mg/ml (Table 2). MIC and MFC of *A. galanga* using macro dilution techniques were employed with extracts made from chloroform, hexane and water. The MIC value of *A. galanga* chloro-form soluble fractions was lower (25 µg/ml) than those observed for other extracts.

Acetoxychavicol acetate and some related phenylpropanoids were isolated from rhizomes of *A. galanga* (Matsuda et al., 2003). This compound showed antifungal activity against fungi tested by Janssen and Scheffer (1985). However, acetoxychavicol acetate was not identified in this study. On the other hand, the main compounds detected in *A. galanga* extract were cinnamic acid and its derivatives. These unsaturated carboxylic acids showed antifungal activities in this study and was similar to Narasimhan et al. (2004). This is the first report of the presence of gingerol in the genus *Alpinia*.

Mode of action of *A. galanga* extracts against *Aspergillus* or other fungi is not completely clear yet. The major molecular targets for phenolic compounds present in *A. galanga* are believed to be proteins (Zhu et al., 2009). According to Wink (2006) most phenolic compounds

(Phenylpropanoides, polyphenols such as flavonoides, catechins, tannins, quinines) interfere with proteins in an unselective way by forming non-covalent bonds and changing the protein conformation leading mostly to inactivation.

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