Malaysian Journal of Medical Sciences, Vol. 9, No. 2, July 2002 (23-33)

ORIGINAL ARTICLE

PRELIMINARY SCREENING OF ENDOPHYTIC FUNGI FROM MEDICINAL PLANTS IN MALAYSIA FOR ANTIMICROBIAL AND ANTITUMOR ACTIVITY

Son Radu* and Cheah Yoke Kqueen

Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

The screening of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeast and fungi was carried out on isopropanol extracts prepared from 121 isolates of endophytic fungi isolated from medicinal plants in Malaysia. Sensitivity was found to vary among the microorganisms. *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Alternaria* sp. were susceptible to extracts from three, two and two isolates of endophytic fungi, respectively. None were found effective against *Salmonella typhimurium*. Sixteen endophytic fungal isolates tested were also found to exhibit antitumor activity in the yeast cell-based assay.

Key words : *endophytic fungi, antimicrobial, antitumor*

Submitted-22.5.2001, Revised-21.8.2001, Accepted-16.11.2001

Introduction

The nature and biological role of endophytic fungi with their plant host is variable. Endophytic fungi are known to have mutualistic relations to their hosts, often protecting plants against herbivory, insect attack or tissue invading pathogens (1-3); and in some instances the endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favors virulence (4-5). In Malaysia, extract from many types of local plants are used in traditional manner for treatments of various ailments (6-7). The question is whether they are produce by the plant itself or as a consequence of a mutualistic relationships with beneficial organisms in their tissue. Many reports showed that in a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antibacterial and antifungal. Thus in this study, we focus on the isolation of endophytic fungi and screening them for bioactivity.

Materials and methods

Isolation of endophytic fungi

A random sample from each plant consisting of asymptomatic leaves and branches was taken. Leaves and branches portion were thoroughly washed in running tap water, after which they were surface sterilized by submerging them in 75% ethanol for 2 min. The branch portions were further sterilized sequentially in 5.3% sodium hypochlorite solution for 5 min, and 75% ethanol for 0.5 min. After drying, each leaf was divided into three segments and placed on potato dextrose agar (PDA) supplemented with 50 mg/l chloramphenicol to suppress bacterial growth. Branch portions were cut to expose their inner tissue and placed on the same medium. All the plates were incubated at 27°C for up to 3 weeks. Emerging fungi were transferred to fresh PDA plates, incubated for 1 week and periodically checked for purity.

Table 1 : Endophytic fungi isolated from medicinal plants

No	Local name	Scientific name	First isolation	Second isolation
1.	Asam jawa	*Tamarindus indica*	1B	b1L
2.	Ati-ati	*Coleus blumei*	2L/B	-
3.	Bangun-bangun/Sapooh	*Coleus camosus*	3L	-
4.	Bawang putih/Garlic	*Alium sativum*	-	-
5.	Bisa ular	*Barieria lupulina*	5L, 5B	b5L
6.	Bunga melur	*Jasminum sambac*	6L, 6B	b6L1, b6L2, b6B
7.	Bunga tahi ayam	*Lantana camara* L	7L1, 7L2, 7B	b7L, b7B
8.	Cekam bumi	*Elephantopus scaber*	8L, 8ML	-
9.	Cekur	*Kaemferia galanga*	9L	b9L
10.	Cekur kuning	*Kaempferia angustifolia*	-	-
11.	Celaka merah	*Plumbago indica*	11L1, 11L2	b11L

Continue from Table 1

12.	Cemperai	*Champerela griffithii*	12L	b12L1, b12L2
13.	Daun kepah	*Rhoeo discolor*	13L1, 13L2	b13L1, b13L2
14.	Ervalanala	*Aerva lanata*	14L	b14L
15.	Gandarusa	*Gendarussa vulgaris*	15L1, 15L2, 15B1, 15B2	b15L
16.	Halia bara	*Zingiber minor*	16L, 16ML	-
17.	Inai	*Lawsonia inermis*	-	b17B
18.	Jarak pagar	*Ricinus communis*	18ML	-
19.	Jarak untut gajah	*Jatropha podagrica*	19L	b19L
20.	Jerangau	*Acorus calamus*	20L, 20ML	b20L
21.	Kadok	*Piper longum*	21L, 21B1, 21B2	b21L
22.	Kayu manis	*Cinnamomum zeylamicum*	22L	b22L
23.	Kemangi	*Ocimum basilicum*	23B	-
24.	Kesum	*Polygonum*	24L1, 24L2	b24L1,

Continue from Table 1

		minus		b24L2
25.	Kucing galak	*Acalypha indica*	-	b25L
26.	Kunyit putih	*Curcuma* sp.	26L, 26B	b26L1, b26L2
27.	Lengkuas padi	*Languas conchigera*	17L1, 27L2	b27L1, b27L2
28.	Lidah buaya	*Aloe vera*	-	-
29.	Lidah mertua	*Sansevaieria trifasciata*	29L	b29L
30.	Mengkudu	*Morinda citrifolia*	30L1, 30L2	b30L1, b30L2
31.	Misai kucing	*Orthosiphon staminae*	-	b31L
32.	Naga Buana	*Phyllanthus pulcher*	-	b32L
33.	Nilam	*Coleus amboinicus*	33L	b33L
34.	Pandan	*Pandanus odons*	-	b34L
35.	Pasak bumi	*Andrographis paniculata*	35L	-
36.	Pegaga segi	*Hydrocotyle*	-	-

Continue from Table 1

		patens		
49.	Subong	*Blumae balsamifera*	-	b49L
50.	Tembaga suasa besar	*Rinum aisiaticum*	50L, 50ML	b50L
51.	Temu hitam	*Curcuma aeruginosa*	-	b51L1, b51L2
52.	Temu merah	*Curcuma phaeocaulis*	-	b52L
53.	Tongkat Ali	*Eurycoma longifolia*	-	b53L
54.	Tulang-tulang	*Euphorbia tirucalli*	-	-
55.	Ubi gadong	*Dioscorea hispida*	55L1, 55L2, 55ML	b55L1, b55L2
56.	Ubi garut	*Maranta arundinacea*	56L	-
57.	Bonglai	*Zingziber cassumunar*	-	B57L
58.	Cekur manis	*Phylanthus frondosus*	-	b58L
59.	Cotet mas	*Fleus jelsoidea*	-	b58L
60.	Karipulei	*Murraya*	-	b60L, b60B

Continue from Table 1

		patens		
49.	Subong	*Blumae balsamifera*	-	b49L
50.	Tembaga suasa besar	*Rinum aisiaticum*	50L, 50ML	b50L
51.	Temu hitam	*Curcuma aeruginosa*	-	b51L1, b51L2
52.	Temu merah	*Curcuma phaeocaulis*	-	b52L
53.	Tongkat Ali	*Eurycoma longifolia*	-	b53L
54.	Tulang-tulang	*Euphorbia tirucalli*	-	-
55.	Ubi gadong	*Dioscorea hispida*	55L1, 55L2, 55ML	b55L1, b55L2
56.	Ubi garut	*Maranta arundinacea*	56L	-
57.	Bonglai	*Zingziber cassumunar*	-	B57L
58.	Cekur manis	*Phylanthus frondosus*	-	b58L
59.	Cotet mas	*Fleus jelsoidea*	-	b58L
60.	Karipulei	*Murraya*	-	b60L, b60B

Continue from Table 1

		koenigii		
61.	Kemunting cina	*Catharantus roseus*	-	b61L
62.	Lada hitam	*Piper nigrum*	-	-
63.	Lemba	*Cucurtingo villosa*	-	b63L
64.	Limau kasturi	*Citrus microcarpa*	-	b64L1, b64L2
65.	Melati	*Telosma cordata*	-	b65L
66.	Mengkudu hutan	*Morinda elliptica*	-	b66L
67.	Pepulut	*Urena lobata*	-	b67L
68.	Pinang makan	*Areca catechu*	-	-
69.	Sambung nyawa	*Gynura procumbeus*	-	b69L1, b69L2
70.	Serai kayu	*Eugenia polyantha*	-	b70L1, b70L2
71.	Taji denak	*Zizyphus oenoplia*	-	b71L
72.	Ulam raja	*Cosmos caudatus*	-	b72L, b72B

No endophytic fungi isolated.

Table 2 : Endophytic fungal isolates showing biological-activity against test organisms.

	Antimicrobial activity (mm)[a]				Antitumor activity (mm)[b]	
Endophytes	**Bs**	**St**	**Sc**	**Al**	**UCS**	**UCK**
12L	15	-	-	-	-	-
19L	19.5	-	-	20	-	-
22L	19.2	-	-	-	-	-
21L2	-	-	13.2	-	-	-
27L1	-	-	13.5	-	-	-
1B	-	-	-	21	-	-
5L	-	-	-	-	8	8.5
24L2	-	-	-	-	8	8
37L	-	-	-	-	9.2	9.1
41L1	-	-	-	-	8.3	8
50ML	-	-	-	-	10	10.3
b34L	-	-	-	-	7.2	7.1
b53L	-	-	-	-	8	8.1
b20L	-	-	-	-	7.5	7.5
b69L2	-	-	-	-	8.1	8
b9L	-	-	-	-	10	10.3
b30L	-	-	-	-	9	9
b7L	-	-	-	-	9.3	9.2
b70L2	-	-	-	-	8.6	8.3
b14L	-	-	-	-	8.2	8.2
b49L	-	-	-	-	8.7	8.5
b29L	-	-	-	-	8.3	8.1

[a] Test microorganisms: Bs, *Bacillus subtilis*; St, *Salmonella typhimurium*; Sc, *Saccharomyces cerevisiae*; and Al, *Alternaria sp*.

[b] Yeast test strain W303-1AY18 containing: plasmid pMR438-CyclinA Δ 24-62 (UCK) or Yep51-SRX5 src (UCS).

- None detected.

Antimicrobial and antitumor activity tests

The endophytic fungi were grown at 27°C with shaking in 5 ml F-4 medium (Glycerol 40 g/l, Soy bean meal 25 g/l, Yeast extract 5 g/l, Corn steep liquor 1 g/l, NaCl 0.5 g/l) and PD-Y medium (Potato dextrose broth 24 g/l, Yeast extract 2 g/l) for 5 days. For extraction, an equal volume of isopropanol was added to the culture broth and vortexed vigorously for 1 min followed by a centrifugation at 3,000 rpm for 10 min. About 80 ml of supernatant was applied per sterile paper disc (5 mm diameter). After drying, the extract impregnated discs were used in a disc diffusion assays using *Alternaria* sp. in potato dextrose agar (PDA), *Bacillus substilis*, *Salmonella typhimurium* in PMg agar, and *Saccharomyces cerevisiae* in YPG agar as test microorganisms for antimicrobial activity. Five milliliter of spore suspension of *Alternaria* sp. grown in vegetable juice (tomato juice, 200 ml $CaCO_3$, 4.5 g; agar, 3.0 g in a total vol. of 300 ml) were used. *S. cerevisiae* was grown in YPG broth (yeast extract, 20 g/l; peptone 20 g/l; glucose, 20 g/l), and the *B. subtilis* and *S. typhimurium* were grown in PMg broth (peptone 10 g/l; $MgSO_4.7H_2O$, 2 g/l). Chloramphenicol (50 mg/ml) and nystatin (100 mg/ml) were used as positive controls.

To prepare assay plate in a 21.5 cm x 21.5 cm square plate, Yeast Nitrogen Base (YNB) broth (1.4 g) and Bacto agar (2.4 g) were dissolved in 150 ml of sterile distilled water. The pH was adjusted to 6.5 prior to autoclaving at 121°C for 15 min. When the agar is about 42°C, the following components were added: 20 ml of 50% galactose, 2 ml of 20% sucrose, 10 ml of 20x concentrated adenine (0.5 mg/ml), 2 ml of 100x concentrated histidine (2 mg/ml), 2 ml of 100x concentrated tryptophan (2 mg/ml), 2 ml of 100x concentrated uracil (2 mg/ml) or 2 ml of 100x concentrated leucine (2 mg/ml) when using the test strain UCK or UCS, respectively; 10x concentrated 4 dropout amino acid (containing each at 10x concentrated of arginine at 240 mg, methionine at 240 mg, tyrosine at 360 mg, isoleucine at 360 mg, lysine at 360 mg, phenylalanine at 600 mg, aspartic acid at 1000 mg, valine at 1500 mg and thymine at 2000 mg per 1000 ml sterile distilled water) and yeast glycerol (30-40%) stock of the yeast test strain W303-1AY18 containing either the plasmids pMR438-CyclinA D 24-62 (UCK) or Yep51-SRX5 src (UCS) (8), and poured into the square plate on a horizontal place. The 5 mm paper disc impregnated with the supernatant described above were placed on the agar plate and incubated at 30°C for 3-4 days. The growth circle around the disk which indicate positive results for anti-tumor activity was measured. Glucose (50%) was used as a positive control. All the screenings procedures were performed twice in duplicates. Inhibition zone (for antimicrobial test) or growth zone (for antitumor test) around the disk of 6 mm or more were defined as positive for biological activity.

Results and discussion

Plants have long provided mankind with a source of medicinal agents, with natural products once serving as source of all drugs (9). Though synthetic chemical also have long been used as active agents in reducing the incidence of plants, animals and humans diseases, they are costly, have potentially harmful effect on the environment and may induce pathogen resistance. Thus, biological controls or the use of microorganisms or their secretions to prevent diseases offer an attractive alternative or supplement to disease management without the negative impact of chemical control.

In natural product discovery programs, typical procedures included isolating microorganisms from samples, growing at various temperatures in a variety of selective or nonselective media and testing the extracts in a spectrum of targeted screens for activity for potential industrial or pharmaceutical applications. For a successful fungal screening, a varied and novel repertoire of either well-known or unexplored fungi is desirable. The most promising trend in isolating new fungi is the move towards investigating novel endophytes, with the idea that unusual endophytes may produce untapped natural products.

A total of 121 endophyte isolates were obtained from 62 of 72 (86.1%) different types of medicinal plants use by the local population in Malaysia (Table 1). The results of this study showed that endophyte fungi were more prevalent in the leaves (110/121 or 90.9%) than the branches. Further and more intensive samplings are necessary to clarify the fungal assemblages of the leaves and branches, as in traditional practice, the local population used mostly the extract from the leaves of the plants (6-7). Though there are still a lot of subjects to be explained in the mutualistic association of endophytic fungi and their plant host, more reports indicated the occurrence of endophytes in plants especially in relation to the possible origin of the plant metabolites detected (10-12). The culture residue of the isopropanol extract of the endophytes

cultures 12L and 22L in F-4 medium and 1B, 19L, 21L2 and 27L1 in PD-Y medium yielded impressive anti-fungal, anti-bacterial or anti-yeast activities. However, only extracts from endophytic fungal cultures of 5L, 24L2, 37L, 41L1, 50ML, b34L1, b53L, b20L, b69L2, b30L, b9l, b70L2, b14L, b49L and b29L in PD-Y broth showed positive activity for anti-tumor in the UCK/UCS yeast cell-based assay (Table 2). The basis of the anti-tumor screening using a yeast cell-based assay was that the hyperactivation of cylcin-dependent-kinase (CDK) resulted in growth arrest of the yeast harboring the genetically engineered recombinant plasmids, and a compound from the extract that can rescue the cyclinA1-induced growth arrest is viewed as a potential anti-tumor candidate.

In this study, we demonstrated that crude extracts from the culture broth of endophytic fungi grown aerobically in PD-Y or F4 medium displayed anti-bacterial, anti-fungal, anti-yeast or anti-tumor activity. These results suggest the presence of either good antimicrobial potency of the extract or of a high concentration of an active principle in the extracts of strains showing positive biological activities. Other endophytic fungal extracts which showed low anti-microbial or anti-tumor activity in the bioassay may have active compounds but probably in smaller amounts and/or the screened crude extracts could yield more potent compounds once they had undergone some purification (13). Also extracts which showed no anti-microbial or anti-tumor activity in the disc-diffusion bioassay may be active against other microbes which were not tested. Looking at the differing activity of test results obtained, additional modes of action should be explored for those isolates that do not have antimicrobial activity, as it is possible that some of these endophytes may produce substances that may ward off microbial infections by stimulating the host immune system rather than by antimicrobial activity. In addition, there is also the possibility that substances present in the extract can stimulate the growth of the microorganisms, as was evident by several isolates showing good bacterial growth forming wide zone of inhibition around the disk, thus counteracting the effect of inhibitory substances.

The observation that antibacterial and antifungal, although in crude extract, were detectable in several isolates may indicate, but not prove, that these isolates produce bioactive substances. In traditional natural products screening programs extracts that are 'hits' in a screen of interest require follow-up analysis, typically involving analytical chemists. This aspect will be further investigated as in any natural product screening, the "referm" problem (rare cultures that produce an activity of interest the first time they are grown often cannot be made to produce that activity again when they are refermented) need to be addressed to enhance production of the secondary metabolites of interest. Therefore, any information and/or research on endophyte-plant symbiosis, such as in this study is of value, especially taking into account the positive biological activity as anti-microbial and anti-tumor agents. Effective extracts could provide potential leads towards the development of novel and environmental friendly biologically active agents.

Acknowledgments

This study was supported by the Malaysian Government through the IRPA grant mechanism and the funds from the Japanese Society for Promotion of Science (JSPS Group 2) under the Large Scale Cooperative Research in the Field of Biotechnology, Subproject on Diversity in Endophytes in Malaysian Plants and Their Ecological and Taxanomic Characteristics.

Correspondence:

Assoc. Prof. Dr. Son Radu
Department of Biotechnology,
Faculty of Food Science and Biotechnology,
University Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.
Tel: 06 – 89486101 ext. 8361; Fax: 03 – 89423552
Email: son@fsb.upm.edu.my

References

1. Siegel, M. R., Latch, G. C. M. and Johnson, M. C. *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: significance and control. *Plant Dis*. 1985; **69**: 179-183.
2. Clay, K. Grass endophytes. In: Fokkema, N. and van den Heuval, J., eds. *Microbiology of the Phyllosphere*. Cambridge University Press, Cambridge, 1986; 392.
3. Yang, X., Strobel, G., Stierle, A., Hess, W. M., Lee, J. and Clardy, J. A fungal endophyte-tree relationship: *Phoma* sp. in *Taxus wallachiana*. *Plant Sci.* 1994; **102**: 1-9.

4. Carroll, G. C. The biology of endophytism in plants with particular reference to woody perennials. In: Fokkema, N. and van den Heuval, J., eds. *Microbiology of the Phyllosphere*. Cambridge University Press, Cambridge, 392 pp. 1986.

5. Bettucci, L. and Saravay, M. Endophytic fungi in *Eucalyptus globulus*: a preliminary study. *Myco. Res*. 1993; **97**: 679-682.

6. Ong, H. C. and Noralina, J. Malay herbal medicine in Gemencheh, Negeri Sembilan, Malaysia. *Fitoterapia* 1998; **70**: 10-14.

7. Ong, H. C. and Nordiana, M. Malay ethno-medico botany in Machang, Kelantan, Malaysia. *Fitoterapia* 1999; **70**: 502-513.

8. Sikder, H., Fukakoshi, M., Nishimoto, T. and Kobayashi, H. An altered nuclear migration into the daughter bud is induced by the cyclin A1-mediated Cdc28 kinase through an aberrant spindle movement in *Saccharomyces cerevisiae*. *Cell Struct, Funct*. 1997; **22**: 465-476.

9. Balandrin, M. F., Kinghorn, A. D. and Farnsworth, N. R. Plant-derived natural products in drug discovery and development. In: Kinghorn, A. D. and Balandrin, M. F., eds. *Human Medicinal Agents from Plants*. American Chemical Society, Washington, D. C. 1993.

10. Strobel, G. A., Hess, W. M., Ford, E. J., Sidhu, R. S. and Yang, X. Taxol from fungal endophytes and the issue of biodiversity. *J. Industr. Microbiol.* 1996; **17**: 417-423.

11. Cacabuono, A. C. and Pomilio, A. B.. Alkaloids from endophyte-infected *Festua argentina*. *J. Ethnopharmacol*. 1997; **57**: 1-9.

12. Rizzo, I., Varsavky, E., Haidukososki, M. and Frade, H. Macrocyclic trichothecence in *Baccharis coridifolia* plants and endophytes and *Baccharis artemisioides* plants. *Toxicon* 1997; **35**: 753-757.

13. Fabry, W., Okemo, P. O. and Ansorg, R. Antibacterial activity of East African medicinal plants. *J. Ethnopharmacol*. 1998; **60**: 79-84.