Proceedings of The 2nd Annual International Conference Syiah Kuala University 2012
& The 8th IMT-GT Uninet Biosciences Conference
Banda Aceh, 22-24 November 2012

Antimicrobial activity of *Phomopsis* sp. ED2, an endophytic fungus isolated from *Orthosiphon stamineus* Benth

[1]Tong Woei Yenn, [1]Darah Ibrahim, and [2]Latiffah Zakaria

[1]Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia, [2]Plant Pathology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia. Coorresponding Author: woeiyenn@yahoo.com

Abstract. This study aimed to investigate the antimicrobial activity of the endophytic fungus *Phomopsis* sp. ED2, which was previously isolated from the flower of the medicinal plant *Orthosiphon stamineus* Benth, in Penang, Malaysia. The 14 days-old fungal cultures were extracted with different organic solvents, include hexane, dichloromethane, ethyl acetate and butanol. The antimicrobial activities of the extracts were tested by disc diffusion assay. Based on the results, most antimicrobial compounds were present in the ethyl acetate fraction, and this extract could significantly inhibit the activity of bacteria, yeasts and fungi. The results also indicate that the antimicrobial compounds were mainly associated with the fungal biomass and all the Gram-positive test bacteria were inhibited by the ethyl acetate extract of the fungal biomass. Notably, methicillin-resistant *Staphylococcus aereus* exhibited a high sensitivity to the extract, with a low minimal inhibitory concentration and low minimal lethal concentration. The isolate also effectively inhibited the growth of the dermatophytes *Microsporum gypseum* and *Trichophyton rubrum*. Preliminary phytochemical screening also demonstrated that the extract contains phenols and steroids which possess antimicrobial activity. These results reveal that the endophytic fungus *Phomopsis* sp. is potential source of novel chemotherapeutic agents.
Keywords: Antimicrobial activity, endophytes, *Phomopsis* sp., *Orthosiphon stamineus*.

Introduction

The mis-usage of antibiotics had caused the threats of antibiotic resistant ”superbugs”, hence a new antibiotics from natural sources are necessary. Endophytes, which occupy a unique biotope with a global estimation up to one million species are a good source of antibiotic compounds (Schulz *et al*., 2002). Endophytes are microorganisms which reside a whole or a part of its life-cycle asymptomically in the living plant tissue without causing any daage to the host plant (Tan and Zou, 2001). They may contribute to the well-being of the host plant by producing bioactive secondary metabolites, thus theyare expected to have good potential as sources of new natural bioactive compounds and plant metabolites (Mourad, 2010).

Phomopsis is a genus that includes over 1000 species classified on the basis of their plant host, and is frequently isolated as an endophyte (Farr *et al.*, 2002). It is also well known for the production of bioactive compounds that exhibit antimicrotubule (Sreekanth *et al*., 2011), antimalarial (Farr *et al*., 2002), antitubercular (Castillo *et al*., 2002), antifungal (Ding *et al*., 2008), herbicidal (Qiu *et al*., 2006), algicidal (Gond *et al*., 2007), anti-inflammatory (Nithya and Muthumary, 2011), antimicrobial, and plant growth regulatory activities (Horn *et al*., 1995). These bioactive compounds include phomopsin, phomopsidin, phomoxanthones, phomoenamide, phomonitroester, diacetylphomoxanthone, phomodiol, diaryl ethers, phomosines, phomalactone, phomopsichalasin, and cytochalasin (Huang *et al*., 2008). Nowadays, Phomopsis sp. Has grawn the attention of researchers becauset they are a potential source of pharmaceutical agents.

Basically, the purpose of this study was to study the antimicrobial activity *Phomopsis* sp. ED2, an endophytic fungus from medicinal herb *O. stamineus* Benth. The antimicrobial activity of the fungal isolate has been tested on various pathogenic test microoganisms including both bacteria, fungi and yeasts.

Materials and Methods

Storage of endophytic fungus

The endophytic fungus previously isolated by Tong *et al*. (2011) from the flower *O. stamineus* Benth was deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (AES) slant and stored at 4^0C. The culture was subculturing on fresh medium every four weeks to ensure the viability of the isolate.

Culture media

Yeast extract sucrose broth (sucrose 40 g/L, yeast extract (AES) 20 g/L and magnesium sulfate 0.5 g/L) supplemented with the aqueous extract of *O. stamineus* was used to cultivate the endophytic isolates in the shake-flask system. The plant extract was prepared by boiling 10 g

of the powdered plant materials in 500 mL distilled water for 30 minutes. The extract was filtered and mixed with freshly prepared culture media and autoclaved at 121 ℃ for 15 min. The aqueous extract of the host plant has been added in the culture media as previous study by Tong *et al*. (2011) showed that the addition of host plant extract in the culture media can significantly enhanced the antimicrobial activity of endophytic fungus.

Fermentation and extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 mL Erlenmeyer flasks containing 100 mL of the broth medium. Both agar plugs were 1 cm in diameter and excised from the 7-days-old fungal culture. The cultures were incubated at 30 ^{0}C with rotational speed of 120 rpm. After 14 days of incubation, the fermented broth and fungal biomass were separated out by centrifugation at 5311 g (Sigma, Model 4K15). Freeze-dried fungal biomass was extracted by soaking in methanol (1:50, w/v) overnight. The filter was then extracted sequentially with hexane, dichloromethane, ethyl acetate and butanol (1:1, v/v). Supernatant of the culture broth was then extracted thrice with equal volume of hexane, dichloromethane, ethyl acetate and butanol (1:1, v/v). The organic extracts were evaporated under reduced pressure.

Test microorganisms

The test microorganisms used in the study included 2 gram-positive bacteria [*Proteus mirabilis* and Methicilin-resistant *S. aureus* (MRSA)], 2 gram-negative bacteria [*Escherichia coli* and *Klebsiella pneumoniae*], 2 yeasts [*Candida utilis* and *Cryptococcus neoformans*] and 2 fungi [*Microsporum gypseum*, and *Trichophyton rubrum*]. The cultures were provided by Industrial Bitechnology Research Laboratory and Plant Pathology Research Laboatory, Universiti Sains Malaysia, Penang, Malaysia. The bacterial cultures were subculturing every two weeks on fresh nutrient agar (NA) slants (Hi-media) and incubated at 37 ^{0}C, whereas the yeasts and fungal cultures were subculturing every four weeks on the fresh potato dextrose agar (PDA) (Hi-media) slants and incubated at 37 ^{0}C for yeasts and 30 ^{0}C for fungi. All the cultures were then kept at 4 ^{0}C until further use. The inoculum was prepared by adding 4 mL of sterile physiological saline to the agar slant, and shake vigorously to get the cell or spore suspension.

Disc diffusion assay

The crude extracts were dissolved in 5% dimethyl sulfoxide (DMSO) and sterilized by filtration with 0.2 µm Milipore filter. Mueller Hinton agar (Hi-media) plate was used for test bacteria, Mueller Hinton agar plate containing 2% dextrose and 0.5 µg/mL methylene blue for test yeasts, and RPMI 1640 (Sigma) agar plates was used for test fungi. RPMI 1640 agar plates were prepared by filter-sterilizing two times (2X) RPMI broth with 0.2 µm Milipore filter and followed with the addition of two times sterile agar after cooling at the time of experiment. Disc diffusion assay was carried out by inoculating 100 µL of suspension containing 10^5 CFU/mL of bacteria, 10^6 CFU/mL of yeasts and 10^6 spores/mL of fungi spreaded on the surface of agar medium. Sterile Whatman antibiotic disc, impregnated with 20 µL of each extracts of 20 mg/mL concentration, were then placed on the surface of inoculated medium. Five percent DMSO was applied as a negative control to detect the solvent effects whereas 30 µg/mL chloramphenicol was used as the positive controls for bacteria, 30 µg/mL Amphotericin B for fungi and yeasts, respectively. The plates were incubated at 30 ^{0}C for 48 to 96 hours for fungi, and at 37 ^{0}C for 24 hours for bacteria and yeasts. The diameter of the clear zones surrounding the disc were measured after the incubation period.

Determination of MIC and MLC

The minimal inhibitory concentration (MIC) was determined by using broth microdilution assay in sterile 96-well microtiter plate and only the test microorganisms that showed significant inhibitory activity on disc diffusion assay were tested. Sterile Muller Hinton broth was used for test bacteria whereas RPMI 1640 medium containing 0.2% dextrose buffered with 0.165 M MOPS to a pH of 7.0 at 25 ^{0}C were used for test yeats and test fungi. The fungal extract was dissolved in 5 % of DMSO to the concentration of 2 mg/mL, which then diluted to the highest concentration to be tested (1 mg/mL) after addition of 100 µL inoculum to achieve final volume of 200 µL. Then serial two fold dilution of the extract was carried out in a concentration range from 500 µg/mL to 15.63 µg/mL. The well containing only 5% DMSO and inoculum was used as the control. The plates were incubated at 30 ^{0}C for 48 to 96 hours for fungi, and at 37 ^{0}C for 24 hours for bacteria and yeasts. After the incubation period, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet salt (INT) (Sigma) dissolved in 99.5% ethanol was added to each well

as a growth indicator for test bacteria and yeasts. The color of INT changed from yellow to purple indicates the microbial growth. The MIC value was recorded as the lowest concentration of extract that prevents any visible growth of teh test mucroorganism. To determine minimal lethality concentration (MLC) of the extract, the sample from each well was taken and viable cell count was conducted to judge the viability. The MLC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

Results and Discussion

The antimicrobial activity of *Phomopsis* sp. ED2 in a disc-diffusion assay is shown in table 1 and table 2. Generally, the extract prepared from the fungal biomass exhibited better antimicrobial activity. This indicates that the antimicrobial compounds were mainly associated with the fungal biomass. The results indicate that all the gram-positive test bacteria were inhibited by the ethyl acetate extract of the fungal biomass. These bacteria were more susceptible than the gram-negative bacteria to the fungal extract where only one gram-negative bacteria were inhibited by the extract. The difference in sensitivity can be due to the morphological differences between these bacteria (Guo *et al*., 2011). The cell wall of gram-negative bacteria has an outer phospholipid membrane and has lipopolysaccharide components. This makes the cell wall of gram-negative bacteria less permeable to lipophilic solutes than the cell wall of bacteria.

The results are in agreement with those obtained from previous studies as the antimicrobial compounds from *Phomopsis* sp. in those studies also were mainly present in the ethyl acetate extract (Rukachaisirikul *et al.*, 2007; Huang *et al.*, 2008). This finding also indicates that the antimicrobial compounds were semi-polar in nature and can be extracted using ethyl acetate and dichloromethane. None of the antimicrobial compounds was present in the highly polar (butanol) and non-polar (hexane) fraction.

The MIC and MLC vaues of the extract on various test microorganisms were presented in table 3 and table 4. It is worth to note that the ethyl acetate extract of the fungal biomass exhibited significant inhibitory activity on MRSA. The MIC and MLC values of this extract were similar for MRSA, thereby mirroring the high sensitivity of MRSA towards the antibacterial compounds in the extract (Ding *et al*., 2008). The low MLC values of the extract for these multidrug-resistant pathogens can therefore validate, to some extent, the use of endophytes from medicinal plants as a source of extremely novel chemotherapeutic agents.

Table 1. Antimicrobial activity of the various extracts from fungal biomass on disc diffusion assay. The results were recorded according to the following scales: +++≥15mm, ++=10-14mm, +≤9mm, -= no antimicrobial activity

Test microorganisms	Diameter of inhibition zones (mm)			
	Hexane	Dichloromethane	Ethyl acetate	n_Butanol
P. mirabilis	-	+++	+++	-
MRSA	-	-	+++	-
E. coli	-	-	-	-
K. pneumoniae	-	-	+++	-
C. utilis	-	-	+	-
C.neoformans	-	-	+	-
M. gypseum	-	-	++	-
T. rubrum	-	-	++	-

Table 2. Antimicrobial activity of the various extracts from fermentative broth on disc diffusion assay. The results were recorded according to the following scales: +++≥15mm, ++=10-14mm, +≤9mm, -= no antimicrobial activity

Test microorganisms	Diameter of inhibition zones (mm)			
	Hexane	Dichloromethane	Ethyl acetate	n_Butanol
P. mirabilis	-	-	++	-
MRSA	-	-	-	-
E. coli	-	-	-	-
K. pneumoniae	-	+++	+++	-
C. utilis	-	-	-	-
C.neoformans	-	-	-	-
M. gypseum	-	-	++	-
T. rubrum	-	-	++	-

Only the ethyl acetate extract of the fungal biomass exhibited inhibitory activity on both test yeats, *C. utilis* and *C. albicans*. However, the anti-yeast activity was relatively weak as the size of inhibition zones were small, less than 9mm in diameter. Besides, the activity was only fungistatic due to the high MLC values. This is probably due to the similarity of eukaryotic characteristics in the endophytic fungus and test yeasts (Castillo *et al*, 2008).

Both of the test fungi were inhibited by *Phomopsis* sp. in the disc diffusion assay. The ethyl acetate extract of the fungal biomass and the fermentative broth exhibited antifungal activity for *M. gypseum* and *T. rubrum,* however the activity exhibited by the ethyl acetate extract from fungal biomass was relatatively better. It is very important to note the potential of *Phomopsis* sp. in inhibiting the growth of dermatophytic fungi. Besides, the activity was fungicidal due to the low MLC values obtained.

Table 3. The MIC and MLC values of the crude extract prepared from fungal biomass on broth microdilution assay

Test microorganisms	Concentration of extract (µg/mL)			
	Dichloromethane		Ethyl Acetate	
	MIC	MBC	MIC	MBC
P. mirabilis	250	500	62.5	125
MRSA	-	-	125	125
K. pneumoniae	-	-	62.5	250
C. utilis	-	-	250	>1000
C.neoformans	-	-	500	>1000
M. gypseum	-	-	125	1000
T. rubrum	-	-	500	1000

Table 4. The MIC and MLC values of the crude extract prepared from fermentative broth on broth microdilution assay

Test microorganisms	Concentration of extract (µg/mL)			
	Dichloromethane		Ethyl Acetate	
	MIC	MBC	MIC	MBC
P. mirabilis	250	500	62.5	125
K. pneumoniae	-	-	62.5	125
M. gypseum	-	-	250	1000
T. rubrum	-	-	500	1000

Conclusions

The study showed that *Phomopsis* sp. ED2, an endophytic fungus from medicinal herb *O. stamineus* exhibited significant antimicrobial activites on various test microorganims. Hence further studies should be conducted to identify the bioactive compounds present in the crude extract.

Acknowledgements

The authors are thankful to National Science Fellowship (NSF) and USM post-graduate research grand scheme for the research grant and financial support in this study.

References

Castillo, U. F., Strobel, G. A., Ford, E. J., Hess, W. M., Porter, H., Jensen, J. B., Albert, H., Robinson, R., Condron, M. A. M., Teplow, D. B., Stevens, D. and Yaver, D. 2002. Munumbicins, wide spectrum antibiotics produced by *Streptomyces* NRRL 30562, endophytic on *Kennedia nigriscans.* Microbiology, 148: 2675-2685.

Ding, G., Liu, S., Guo, L., Zhou, Y. and Che, Y. 2008. Antifungal metabolites from the plant endopytic fungus *Pestalotiopsis foedan*. Journal Natural Products, 71: 615-618.

Farr, D.F., Castlebury, L.A. and Rossman, AY. 2002. Morphological and molecular characterization of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States. Mycologia, 94: 494-504.

Gond, S. K., Verma, V. C., Kumar, A., Kumar, V. And Kharwar, R. N. 2007. Study of endophytic fungal community from different parts of *Aegle marmelos* Correae (Rutaceae) from Varanasi (India). World Journal of Microbiology and Biotechnology, 23: 1371-1375.

Guo, Z., Hua, R., Bai, Y., Wu, X., Cao, H., Li, X., Wu, Y. and Tang, J. 2011. Screening and

evaluation of antiphylopathogenic activity of endophytic fungi from live foliages of *Ginkgo biloba* L. African Journal of Microbiological Research, 5: 1686-1690.

Horn, W.S., Simmonds, M.S.J., Schwartz, R.E. and Blaney, W.M. 1995. Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. Tetrahedron, 51: 3969-3978.

Huang, Z., Cai, X., Shao, C., She, Z., Xia, X., Chen, Y., Yang, J., Zhou, S. and Lin, Y. 2008. Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry, 69: 1604-1608.

Mourad, M. H. E.S. 2010. New bioactive compounds from *Verticillium alboatrum* and *Verticillium leptobactrum.* Australian Journal of Basic and Applied Sciences, 4: 2166-2175.

Qiu, Z. Q., Cao, L. X., Tan, H. M.and Zhou, S. M. 2006. Isolation and characterization of endophytic *Stretomyces* sp. S5 with herbicidal activity from tomato roots. Chinese J ournal of Agricultural Biotechnology, 3: 7-12.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J. and Kirtikara, K. 2007. Metabolites from endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry ,69(3): 783-787.

Schulz, B., Boyle, C., Draeger, S., Rommert, A.K. and Krohn, K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycology Research, 106(9): 996-1004.

Sreekanth, D., Sushim, G. K., Syed, A., Khan, B. M. and Ahmad, A. 2011. Molecular and morphological characterization of a taxol-producing endophytic fungus, *Gliocladium* sp. from *Taxus baccata*. Microbiology, 39: 151-157

Tan, R.X. and Zou, W.X. 2001. Endophytes: a rich source of functional metabolites. Natural Product Reports, 18: 448-459.

Tong, W.Y., Darah, I. and Latiffah, Z. 2011. Antimicrobial activities of endophytic fungal isolates from medicinal herb *Orthosiphon stamineus* Benth. Journal of Medicinal Plants Research, 5: 831-836.