The Thai Journal of Pharmaceutical Sciences

Volume 38
Issue 0 *supplement issue*

Article 11

1-1-2013

# ANTILEUKEMIC ACTIVITY AND SECONDARY METABOLITES OF AN ENDOPHYTIC FUNGUS PHOMOPSIS SP. FROM ARTEMISIA ANNUA

Punvisa Ngankaranatikarn

Taksina Chuanasa

Nongluksna Sriubolmas

Khanit Suwanborirux

Thai J. Pharm. Sci. Vol. 38 (Suppl.) 2013

# ANTILEUKEMIC ACTIVITY AND SECONDARY METABOLITES OF AN ENDOPHYTIC FUNGUS *PHOMOPSIS* SP. FROM *ARTEMISIA ANNUA*

Punyisa Ngankaranatikarn[1], Taksina Chuanasa[1], Nongluksna Sriubolmas[2] and Khanit Suwanborirux[1,*]

[1]*Center for Bioactive Natural Products from Marine Organisms and Endophytic Fungi (BNPME), Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.*
[2]*School of Pharmacy, Eastern Asia University, Pathumthani 12110, Thailand.*

**KEYWORDS:** *Phomopsis* sp., endophytic fungus, antileukemic activity, *Artemisia annua*

## INTRODUCTION

Endophytic fungi are symbiotic microorganisms living within tissues of the plant hosts without causing noticeable diseases. These microorganisms are known as rich sources of bioactive secondary metabolites.[1] Particularly, fungi in the genus *Phomopsis* were commonly found in medicinal plants and reported to produce various secondary metabolites exhibiting interesting bioactivities including cytotoxicity,[2-4] antimicrobial activity[5] and antimycobacterial activity.[6] The metabolites with diverse structures from *Phomopsis* were revealed to possess cytotoxic activity; for example, oblongolides, the hexaketide γ-lactone, from *P. oblongna* and dicerandrols, the xanthone dimers, from *P. longicolla* exhibiting cytotoxicity against human epidermal carcinoma (KB) and human breast cancer (BC) cell lines,[2,3] the cadinane sesquiterpenes from *P. cassiae* showing significant cytotoxic activity against human cervical cancer (HeLa) cell line.[4]

The endophytic fungus *Phomopsis* sp. AANN8 was isolated from the twigs of the Thai medicinal plant *Artemisia annua* L. (Family Asteraceae). The crude ethyl acetate extract from a fermentation broth of this fungus exhibited selective and strong antileukemic activity against human acute monocytic leukemia (THP-1) cell line. Therefore, study of secondary metabolites from this endophytic fungus and their antileukemic activity should be very interesting for anticancer drug development.

## MATERIALS AND METHODS

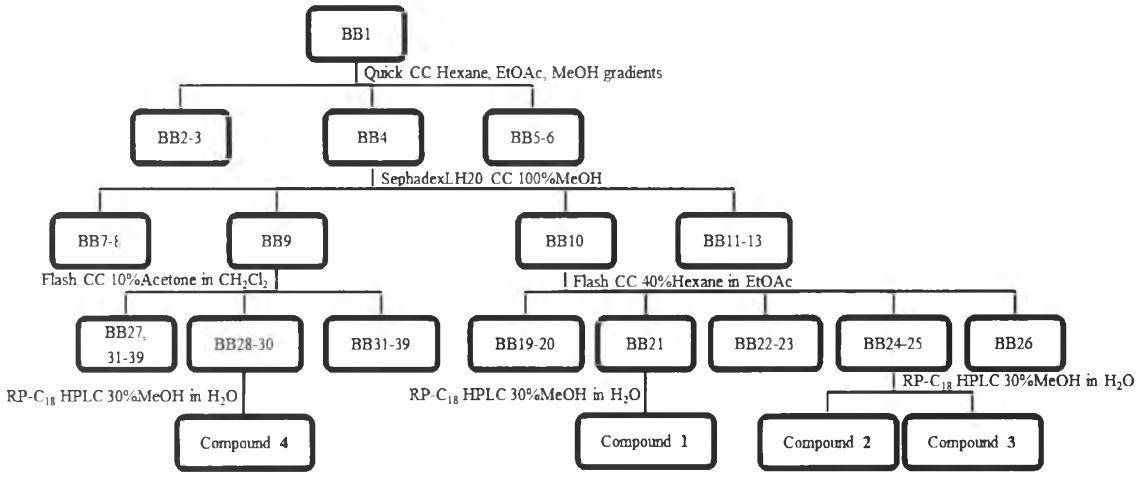
***General procedures*** $^{1}$H-NMR (300 MHz), $^{13}$C-NMR (75 MHz), DEPT, and 2D NMR spectra were taken on a Bruker Fourier 300 NMR spectrometer. Chemical shift values were reported with respect to the residual solvent signals ($\delta_H$7.26/$\delta_C$77.0 for $CDCl_3$ and $\delta_H$2.05/$\delta_C$28.9 for acetone-$d_6$). High-resolution mass spectra were performed on an electrospray ionization JMS-700 (JEOL) instrument with a direct inlet system operating at 70eV. A HPLC system equipped with a Shimadzu LC20AB pump and a LiChroSpher® RP-18 column (250 x 10 mm, 10 μm, Merck) and a SPD-20A UV/Vis detector at 210 nm was used. TLC analysis was carried out using silica gel 60 $F_{254}$ on aluminium sheet (Merck) and spots were visualized under UV light at 254 nm and spraying with 5%anisaldehyde in $H_2SO_4$ followed by heating. Sephadex LH20, silica gel 60 (70-230 and 230-400 mesh, Merck) were used for gel filtration, flash and quick column chromatography, respectively.

***Fungal material*** The fungus *Phomopsis* sp. AANN8 was isolated from the twigs of *A. annua* grown in Kanchanaburi Province, Thailand. The twigs were washed under running sterile distilled water and then air-dried. The cleaned twigs were cut into pieces of 5 cm in length then surface sterilized by 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min and sterile distilled water for three times (1 min, each). The sterilized samples were cut into small pieces using a sterile blade and placed on a sterile water agar plate. All samples were subsequently incubated at 25°C. The hyphal tip of endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile potato dextrose agar (PDA) plate. After incubation at 25°C for 7 days, culture purity was determined by colony morphology.

***Fermentation and extraction*** The endophytic fungus was grown on PDA plates at 25°C approximately for 7 days depending on growth rate. Six pieces (1 x 1 $cm^2$) of the grown culture were prepared and then inoculated into a 1000 ml erlenmeyer flask containing 200 ml of yeast extract sucrose (YES) broth. After incubation at 25°C for 21 days under stationary condition, the fungal culture (total 40 l) was separated into mycelial and filtrate parts. The filtrate broth was extracted by partition with ethyl acetate three times. The combined ethyl acetate phase was evaporated to dryness under reduced pressure to yield the crude ethyl acetate extract (BB1, 6.0 g).

***Isolation*** The crude extract BB1 was isolated by antileukemic assay-guided fractionation as shown in **Scheme 1**. The crude ethyl acetate extract was fractionated into five fractions (BB2-BB6) by a silica gel quick column with gradients of hexane/dichloromethane, hexane/ethyl acetate and methanol. Fraction

BB4 was subjected to Sephadex LH-20 gel filtration chromatography elution with methanol to provide seven fractions (BB7-BB13). Fraction BB10 was separated by a silica gel column using 40%hexane in ethyl acetate as a mobile phase to provide eight fractions (BB19-BB26). Fraction BB21 was purified by semi-preparative HPLC using 30%methanol in $H_2O$ as a mobile phase, flow rate 3 ml/min to yield compound **1** (9.5 mg). Fractions BB24 and BB25 were pooled and subjected to semi-preparative HPLC using 20% methanol in $H_2O$ as a mobile phase, flow rate 3 ml/min to give compounds **2** (49.9 mg) and **3** (8.2 mg). Fraction BB9 was subjected to silica gel column chromatography elution with 10%acetone in dichloromethane to provide nine fractions (BB27-BB35). Fractions BB28-30 were pooled and purified by a semi-preparative HPLC column using 30%methanol in $H_2O$ as a mobile phase, flow rate 2 ml/min to yield compound **4** (6.9 mg).




**Scheme 1.** Extraction and isolation of the ethyl acetate extract of *Phomopsis* sp. AANN8.
Compound **1**: white crystals, $^1$H NMR (acetone-$d_6$) δ (ppm): 8.15 (1H, s, 1-OH), 7.03 (2H, d, $J$ = 8.5 Hz, 3-H and 5-H), 6.72 (2H, d, $J$ = 8.5 Hz, 2-H and 6-H), 3.69 (1H, s, 2′-OH), 3.66 (2H, t, $J$ = 7.2 Hz, 2′-H), 2.69 (2H, t, $J$ = 7.2 Hz, 1′-H); $^{13}$C NMR (acetone-$d_6$) δ (ppm): 156.5 (s, 1-C), 131.6 (s, 4-C), 130.6 (s, 3-C and 5-C), 115.8 (s, 2-C and 6-C), 64.2 (s, 2′-C), 39.4 (s, 1′-C); HR-EIMS *m/z* 138.0680 (calcd for $C_8H_{10}O_2$, 138.0681)

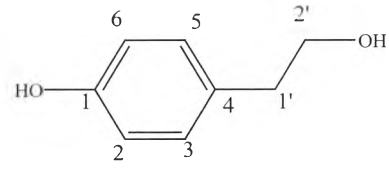
***In vitro antileukemic activity assay*** The crude extract and fractions were evaluated for their antileukemic activity by sulforhodamine B (SRB) colorimetric assay[7] using the human acute monocytic leukemia (THP-1) cell line. Ellipticine was used as the positive control. The crude extract and fractions with the final concentration of 20 µg/ml exhibiting antileukemic activity against THP-1 cell line (over 55% inhibition) were further tested for the effective concentrations that inhibited 50% of THP-1 cell growth ($EC_{50}$) and compared with ellipticine, which its $EC_{50}$ was at 19.46 µg/ml.

## RESULTS AND DISCUSSION

Compound **1** was obtained as white crystals from the culture broth of the endophyte, *Phomopsis* sp. AANN8. The HR-EIMS of **1** displaying a molecular ion at m/z 138.0680 indicated its molecular formula of $C_8H_{10}O_2$ with 4 degrees of unsaturation. The $^1$H NMR data of **1** showed two doublets of four aromatic protons at $\delta_H$7.03 and 6.72 with an *ortho* coupling constant of 8.5 Hz indicating a 1,4-disubstituted aromatic ring and a pair of two-proton triplets of coupled methylenes at $\delta_H$3.66 and 2.69. The $^{13}$C NMR and DEPT spectra of **1** confirmed the 1,4-disubstituted phenyl moiety by a pair of two-carbon signals of aromatic methane carbons at $\delta_C$130.6 and 115.8, one oxygenated quaternary aromatic carbon at $\delta_C$156.5 and one quaternary carbon at $\delta_C$131.6 as well as the ethylene moiety by two methylene carbons at $\delta_C$64.2 and 39.4. The $^1$H and $^{13}$C NMR data of **1** were shown in **Table 1**. Compound **1** was identified as 4-hydroxyphenethyl alcohol or tyrosol by comparison of the $^1$H and $^{13}$C NMR data with the literature.[8] This is the first report of tyrosol obtained from the fugus *Phomopsis*. Tyrosol was previously isolated from plants, such as *Acorus gramineus*[8] and *Olea europea*[9] as well as microbes, such as *Neofusicoccum parvum*[10] and *Glomerella cingulate*[11] The compound was reported to exhibit various bioactivities such as antioxidant[12], anti-inflammatory[13] and neuroprotective[14].

**Table 1.** $^{1}H$ and $^{13}C$ NMR data for **1** in acetone-$d_6$ (δ in ppm).

| Position | $\delta_H$, *mult.* (*J* in Hz) | $\delta_C$ |
|---|---|---|
| 1 | - | 156.5 |
| 2, 6 | 6.72, *d* (2H, 8.5) | 115.8 |
| 3, 5 | 7.03, *d* (2H, 8.5) | 130.6 |
| 4 | - | 131.6 |
| 1′ | 2.69, *t* (2H, 7.2) | 39.4 |
| 2′ | 3.66, *t* (2H, 7.2) | 64.2 |
| 1-OH | 8.15, *br* | - |
| 2′-OH | 3.69, *br* | - |




Tyrosol (**1**)

Antileukemic activity of the ethyl acetate extract (BB1) and fractions (BB2-BB6, BB8-BB10) against THP-1 cell line was presented as percentage inhibition of THP-1 cell growth and $EC_{50}$ values in **Table 2**. The extract showed more potent antileukemic activity than ellipticine. After fraction BB1 was fractionated by a silica gel column, the obtained fraction BB4 presented higher antileukemic activity than BB1. Unexpectedly, the antileukemic activity of fractions BB7-BB13 separated from fraction BB4 dramatically decreased. This result suggested that there might be complexing conditions in fraction BB4 influencing its antileukemic activity, for example some co-factors required for the cytotoxicity were disappeared during the separation or several compounds would be synergistically involved in such a bioactivity.

**Table 2.** Inhibition percentages of THP-1 cell growth and $EC_{50}$ values

| Fraction | %inhibition (at 20 µg/ml) | $EC_{50}$ (µg/ml) |
|---|---|---|
| Ellipticine | nt | 19.46 |
| BB1 | 59.80 | 17.11 |
| BB4 | 85.33 | 0.70 |
| BB8 | 1.97 | nt |
| BB9 | 0.53 | nt |
| BB10 | 52.98 | nt |

nt = not tested

## CONCLUSION

The crude ethyl acetate extract from the fermentation broth of the endophytic fungus, *Phomopsis* sp. AANN8, exhibited antileukemic activity against THP-1 cell line. After separation by antileukemic assay-guided fractionation, four pure compounds (**1-4**) were obtained and compound **1** was identified as tyrosol. Further steps are structure elucidation of other three compounds and moreover, isolations are required to obtain more compounds from fraction BB4. Once isolation and structure elucidation are achieved, antileukemic activity of pure compounds will be evaluated.

**ACKNOWLEDGEMENTS**

This project is supported by BNPME and Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The mass spectrum was measured by Professor Naoki Saito at Meiji Pharmaceutical University. The fungal material and antileukemic activity assay was prepared by Assistant Professor Dr. Suthep Wiyakrutta at Department of Microbiology, Faculty of Science, Mahidol University.

**REFERENCES**

1. Strobel G., Daisy B., Castillo U., and Harper J. Natural products from endophytic microorganisms. Journal of Natural Products, 2004 (67): 257-268.
2. Bunyapaiboonsri T., Yoiprommarat S., Srikitikulchai P., Srichomthong K., and Lumyong S. Oblongolides from the endophytic fungus *Phomopsis* sp. BCC9789. Journal of Natural Products, 2010 (73): 55-59.
3. Clardy J., and Wagenaar M.M. Dicerandrols, New antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an endangered mint. Journal of Natural Products, 2001 (64): 1006-1009.
4. Silva G.H., Teles H.L., Zanardi L.M., Young M.C.M., Eberlin M.N., Hadad R., Pfenning L.H., Costa-Neto C.M., Castro-Gamboa I., Bolzani V.S., and Araujo A.R. Cadinane sesquiterpenoids of *Phomopsis cassia*, an endophytic fungus associated with *Cassia spectabilis* (*Leguminosae*). Phytochemistry, 2006 (67): 1964-1969.
5. Huang Z., Cai X., Shao C., She Z., Xia X., Chen Y., Yang J., Zhou S., and Lin Y. Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry, 2008 (69): 1604-1608.
6. Rukachaisirikul V., Sommart U., Phongpaichit S., Sakayaroj J., and Kirtikara K. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry, 2008 (69): 783-787.
7. Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J.T., Bokesch H., Kennt S., and Boyd M.R. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute, 1990 (82): 1107-1112.
8. Park C.H., Kim K.H., Lee I.K., Lee S.Y., Choi S.U., Lee J.H. and Lee K.R. Phenolic constituents of *Acorus gramineus*. Archives of Pharmacal Research, 2011 (34): 1289-1296.
9. Capasso R., Cristinzio G., Evidente A. and Scognamiglio F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochemistry, 1992 (31): 4125-4128.
10. Evidente A., Punzo B., Andolfi A., Cimmino A., Melck D. and Luque J. Lipophilic phytotoxins produced by *Neofusicoccum parvum*, a grapevine canker agent. Phytopathologia Mediterranea, 2010 (49): 74-79.
11. Guimaraes D.O., Borges W.S., Kawano C.Y., Riberio P.H., Goldman G.H., Nomizo A., Thiemann O.H., Oliva G., Lopes N.P. and Pupo M.T. Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*. FEMS Immunology and Medical Microbiology, 2008 (52): 134-144.
12. Puerta R., Dominguez E.M., Gutierrez V.R., Flavill J.A. and Hoult R.S. Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. Life Sciences, 2001 (69): 1213-1222.
13. Puerta R., Gutierrez V.R. and Hoult R.S. Inhibition of leukocyte 5- lipoxygenase by phenolics from virgin olive oil. Biochemical Pharmacology, 1999 (57): 445-449.
14. Bu Y., Rho S., Kim J., Kim M.Y., Lee D.H., Kim S.Y., Choi H. and Kim H. Neuroprotective effect of tyrosol on transient focal cerebral ischemia in rats. Neuroscience Letters, 2007 (414): 218-221.