Diels-Alder Type Adducts from Hairy Root Cultures of *Morus macroura*

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Abstract – Three Diels-Alder type adducts, guangsangon E (1), chalcomoracin (2) and sorocein I (3) were isolated from hairy root cultures of *Morus macroura*. The structures of the isolated compounds (1-3) were determined by spectroscopic method (NMR and MS), and spectral comparison to literature. Cytotoxic activities of the isolated compounds (1-3) were investigated against P-388 murine leukemia cell line. Guangsangon E (1) showed the most potent cytotoxicity against P-388 murine leukemia cell line with IC50 value of 2.75 ± 0.32 µg/mL. To the best of our knowledge, guangsangon E (1) and sorocein I (3) were reported for the first time from the tissue cultures of *M. macroura*.

Keywords – Diels-Alder type adduct, *Morus macroura*, tissue cultures, cytotoxicity

Introduction

*Morus macroura*, locally known as ‘Andalas’, is a rare plant found and native to the Malesian region.1 It is a tree growing up to 35 m and found at altitudes of 900 - 1600 m.1 In the region, the plant has been valued for the high quality of the wood due to its resistance to insects. Unfortunately, destruction of Indonesian forest threatens the existence and future study of this plant, especially the investigation of secondary metabolites. Plant tissue culture is a method that can be applied to solve the obstacle in investigating compounds in this plant.

Previous phytochemical studies on *M. macroura* revealed the isolation of Diels-Alder type adducts.2-7 Two new benzofuran derivatives, macourins A and B, were also isolated from this plant.8 Our previous works on the chemical constituents of this plant have resulted in the isolation of other phenolic compounds, including andalasin A, andalasin B, quercimeritrin, moracin B, moracin P, oxyresveratrol, and mulberroside C.9,10 Furthermore, our work on hairy root cultures of *M. macroura* afforded chalcomoracin and mulbereofuran P, Diels-Alder type adducts.11

Hairy root culture is one of the interesting methods in the plant tissue cultures since the culture able to grow fast and produce secondary metabolites as well. In our previous work, hairy root cultures of *M. macroura* were successfully produced via transformation of the plant leaves using *Agrobacterium rhizogenes*.11 Furthermore, the effect of *Saccharomyces cerevisiae* extract on production of secondary metabolites of *M. macroura* hairy root cultures had been also investigated.11 In continuation of our studies on these elicited hairy root cultures, here we report the isolation and identification of Diels-Alder type adducts from the methanol extract of the cultures. The isolated compounds (1 - 3) were tested for cytotoxicity against P-388 murine leukemia cell line.

Experimental

General experimental procedures – IR spectra were recorded on a Perkin Elmer spectrum One FT-IR spectrometer. UV spectra were recorded on a Varian Corry 100 Conc spectrometer. NMR spectra were recorded on JEOL JNM A 5000 at 400 MHz (1H) and 100 MHz (13C). MS spectrum of compound 1 was recorded on an ESI-TOF Waters Premier LCT XE mass spectrometer. Meanwhile, MS spectra of compound 2 and compound 3 were recorded using an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex). Vacuum liquid chromatography was used for column chromatography. The13C NMR spectra were acquired at 75 MHz in CD3OD.

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roots of *M. macroura* (35.5 g) were macerated with MeOH yielding 7.9 g of dried extract. The MeOH extract was fractionated with vacuum liquid chromatography (hexane-EtOAc, gradient concentrations) into seven fractions (A-H). Fraction C was subjected to silica gel radial chromatography (EtOAc-chloroform, 2:8) yielding 7 fractions. Fraction C7 was successively purified by Sephadex LH-20 column chromatography and flash silica gel column chromatography (hexane-acetone, 6:4), afforded compound 1 (8 mg) and compound 2 (20 mg). Fraction E was successively purified by silica gel radial chromatography (chloroform-methanol, 9:1) and flash silica gel column chromatography (hexane-acetone, 6:4) yielding compound 3 (5 mg).

*Guangsongon E* (1): Brown amorphous gum; UV (MeOH) $\lambda_{\text{max}}$ (log ε) = 203 (7.45), 225 (7.34), 282 (5.61), 323 (4.93) nm; IR $\nu_{\text{max}}$ (KBr) = 3445, 2927, 1619, 1497, 1461 cm$^{-1}$; $^1$H NMR (500 MHz, acetone-$d_6$): $\delta$ (ppm) = 1.56 (3H, s, H-24''), 1.67 (3H, s, H-25''), 1.89 (3H, s, H-7''), 2.07 (1H, m, H-6a''), 2.46 (1H, m, H-6b''), 3.19 (2H, $d, J = 7.0$ Hz, H-21''), 3.67 (1H, m, H-5''), 4.38 (1H, br s, H-3''), 4.68 (1H, dd, $J = 6.5$, 7.0 Hz, H-I''), 5.12 (1H, t, $J = 7.0$ Hz, H-22''), 5.55 (1H, s, H-2''), 6.14 (1H, dd, $J = 1.5$, 8.5 Hz, H-19''), 6.26 (1H, d, $J = 1.5$ Hz, H-17''), 6.33 (1H, d, $J = 2.0$ Hz, H-4''), 6.42 (1H, d, $J = 8.5$ Hz, H-13''), 6.81 (1H, s, H-7'), 6.83 (1H, d, $J = 2.0$ Hz, H-2'), 6.83 (1H, d, $J = 2.0$ Hz, H-6'), 6.88 (1H, d, $J = 8.5$ Hz, H-20''), 7.04 (1H, s, H-3), 7.45 (1H, s, H-4), 8.03 (1H, d, $J = 8.5$ Hz, H-14''), 13.03 (1H, s, HO-10''); $^{13}$C NMR (125 MHz, acetone-$d_6$): $\delta$ (ppm) = 17.9 (CH$_3$, C-25''), 22.3 (CH$_3$, C-21''), 23.8 (CH$_3$, C-7''), 25.9 (CH$_3$, C-24''), 33.5 (CH, C-5''), 37.4 (CH$_3$, C-6''), 37.9 (CH, C-3''), 48.5 (CH, C-4''), 97.2 (CH, C-7), 102.6 (CH, C-3), 103.4 (CH, C-17''), 103.8 (CH, C-4''), 103.8 (CH, C-2'), 103.9 (CH, C-6), 107.3 (CH, C-13''), 107.6 (CH, C-19''), 115.4 (C-11''), 115.8 (C-9''), 121.9 (C-3a), 123.2 (C-15''), 123.6 (CH, C-4), 123.8 (CH, C-22''), 124.5 (CH, C-2''), 125.6 (C-5), 127.1 (C-8''), 129.9 (CH, C-20''), 129.8 (C-1'), 130.6 (CH, C-14''), 131.2 (C-23''), 135.2 (C-1''), 155.1 (C-7a), 155.1 (C-16''), 155.3 (C-2), 156.4 (C-6), 157.3 (C-18''), 159.8 (C-3'), 159.8 (C-5'), 161.9 (C-12''), 163.9 (C-10''). HRESI-MS: m/z 649.2426 (calcld. 649.2438 for C$_{30}$H$_{47}$O$_2$). The data were accordance with the literature.$^3$

*Chalcomoracin* (2): Yellow amorphous gum; UV (MeOH) $\lambda_{\text{max}}$ (log ε) = 203 (7.45), 319 (7.49), 333 (3.84) nm; IR $\nu_{\text{max}}$ (KBr) = 3349, 2913, 1699, 1419, 1440 cm$^{-1}$; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ (ppm) = 1.56 (3H, s, H-25''), 1.70 (3H, s, H-24''), 1.95 (3H, s, H-7''), 2.21 (1H, m, H-6a''), 2.52 (1H, m, H-6b''), 3.25 (2H, d, $J = 6.9$ Hz, H-21''), 3.75 (1H, t, $J = 4.5$ Hz, H-5''), 4.11 (1H, br s, H-
were in agreement with the literature.\textsuperscript{14} Cytotoxicity assay – Cytotoxicity assay against P388 murine leukemia cell was carried out based on published reports.\textsuperscript{15,16} The leukemia cells at a density of $3 \times 10^4$ cell/mL, were plated in 96-well cultures dishes and incubated at 37°C in humidified CO$_2$ incubator (5%) for 24 h. Guangsangon E (1), chalcomoracin (2) and sorocoein I (3) were first dissolved in DMSO at the required concentrations and prepared subsequently in six desirable concentrations (100 – 0.1 μg/mL) using PBS (phosphoric buffer solution, pH = 7.30-7.65). The prepared samples were added into the sample wells. Meanwhile, the control wells received only DMSO and PBS. Afterward the cell cultures were incubated again for 48 h in 5% CO$_2$ incubator at 37°C. After the incubation, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added and incubated for 4 h. In the next step, MTT-stop solution containing sodium dodecyl sulphate (SDS) were added, and then the last incubation for 24 h was conducted. Cell survival was evaluated with a multi-well scanning spectrophotometer (ELX 800 UV, universal Microplate Reader, Bio-Tek Instruments, Inc.) at 540 nm. All data are presented as the mean values of triplicates.

\section*{Result and Discussion}

Compound 1 was isolated from fraction C of the hairy root extract of \textit{M. macroura} as a brown amorphous gum. According to the spectroscopic analysis (NMR and MS) and comparing with the literature,\textsuperscript{3} compound 1 was assigned to guangsangon E. The molecular structure of guangsangon E (1) is depicted in Fig. 2. Guangsangon E (1) is a Diels-Alder type adduct derived from a substituted chalcone and a substituted 2-phenylbenzofuran. In our knowledge, this is the first report concerning cytotoxic activity of guangsangon E (1).\textsuperscript{3} The leukemia cells at a density of $3 \times 10^4$ cell/mL, were plated in 96-well cultures dishes and incubated at 37°C in humidified CO$_2$ incubator (5%) for 24 h. Guangsangon E (1), chalcomoracin (2) and sorocoein I (3) were first dissolved in DMSO at the required concentrations and prepared subsequently in six desirable concentrations (100 – 0.1 μg/mL) using PBS (phosphoric buffer solution, pH = 7.30-7.65). The prepared samples were added into the sample wells. Meanwhile, the control wells received only DMSO and PBS. Afterward the cell cultures were incubated again for 48 h in 5% CO$_2$ incubator at 37°C. After the incubation, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added and incubated for 4 h. In the next step, MTT-stop solution containing sodium dodecyl sulphate (SDS) were added, and then the last incubation for 24 h was conducted. Cell survival was evaluated with a multi-well scanning spectrophotometer (ELX 800 UV, universal Microplate Reader, Bio-Tek Instruments, Inc.) at 540 nm. All data are presented as the mean values of triplicates.

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\section*{Conclusion and Discussion}

Compound 2 was isolated and purified from fraction C as a yellow amorphous gum. NMR and MS spectra analysis and data comparison to references\textsuperscript{12,13} concluded that compound 2 is chalcomoracin, a Diels-Alder type.
adduct derived from a dehydroprenylchalcone and a substituted 2-arylbenzofuran. The structure of chalcomoracin (2) is depicted in Fig. 2. Chalcomoracin (2) is a phytoalexin, collected for the first time from diseased mulberry leaves (M. alba). Chalcomoracin (2) has been reported having antibiotic activity via inhibition of enoyl-acyl carrier reductase.

According to the cytotoxic assay, chalcomoracin (2) showed moderate cytotoxicity against P-388 murine leukemia cell line with IC_{50} value of 5.46 ± 0.55 µg/mL. Chalcomoracin (2) was reported possessing moderate cytotoxic activities against five human cancer cell lines, including A549, Bel 7402, BGC 823, HCT-8, and A2780. Recently, molecular mechanism of chalcomoracin (2) as anticancer agent had been reported. Chalcomoracin (2) inhibited the cancer cell growth by promoting paraptosis and triggering oxidative stress via a mitophagy. Therefore, the result of cytotoxic assay of chalcomoracin (2) against P-388 murine leukemia cell line was in accordance with the previous reports and confirmed chalcomoracin (2) as potent anticancer agent.

Compound 3 was isolated as a purple amorphous gum. The structure of compound 3 was determined based on NMR and MS data analysis and spectral comparison to reported work. Compound 3 was assigned as sorocein I, a ketalized Diels-Alder type adduct formed from a dehydroprenylchalcone and a dehydroprenylstilbene. Sorocein I (3) was isolated for the first time from Sorocea ilicifolia. Molecular structure of sorocein I (3) was depicted in Fig. 2. Cytotoxic assay revealed that sorocein I (3) was not active against P-388 murine leukemia cell line.

In this report, three Diels-Alder type adducts, guang-sangon E (1), chalcomoracin (2) and sorocein I (3) were isolated from hairy root cultures of M. macroura. Guang-sangon E (1) and sorocein I (3) were recorded for the first time in this tissue culture. Furthermore, cytotoxic activities of guang-sangon E (1) and sorocein I (3) were reported for the first time as well.
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