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Cytotoxic Activity of Secondary Metabolites from the Bark of Dipterocarpus Confertus Sloot

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ABSTRACT

Four compounds, namely β-sitosterol (1), betulinic acid (2), cinnamic acid (3), and α-viniferin (4) have been successfully isolated from the bark methanol extract of *Dipterocarpus confertus* Sloot. The structures of the isolated compounds have been established on the basis spectroscopic data evidence, and comparison with the published data. In the cytotoxicity study, cinnamic acid (3) and betulinic acid (2) have been found to be very strong active against murine leukemia P388 and vero cells lines with the IC_{50} values of 2.25 and 5.10 µg/mL, respectively, while the other compounds were not active.

Keywords

Dipterocarpaceae; Dipterocarpus confertus; Phenolic; Oligostilbenoid; Terpenoid and Cytotoxic

Academic Discipline And Sub-Disciplines

Pharmacy

SUBJECT CLASSIFICATION

Natural drug discovery

TYPE (METHOD/APPROACH)

Experimental

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INTRODUCTION

Dipterocarpus confertus, which known with local name “Keruing Pungguh” in Indonesia is a species of genus *Dipterocarpus* (Dipterocarpaceae). *Dipterocarpus* is the third largest genus in the family of Dipterocarpaceae with 75 species [1,2]. As other genus in Dipterocarpaceae, *Dipterocarpus* has been known as a rich source of oligostilbenoid type of compounds, as well as other type of phenolic compounds [3,4,5,6,7]. The secondary metabolites of Dipterocarpaceae have withdrawn much attention of many scientists due to the structural complexity, and their biological activities such as antifungal [8,9], anti-HIV [10], cytotoxic [10, 11, 3, 4], anti-inflammatory [12, 13] and antibacterial [14, 15, 7]. Our previous study reported the isolation of phenolic compounds from *Dipterocarpus* as well as their cytotoxic activity [4, 16]. In continuation of our interest in the phytochemical study of phenolic compounds in the new species *Dipterocarpus* plant, we report here the cytotoxicity of four compounds isolated from the bark of *Dipterocarpus confertus*.

EXPERIMENTAL

General experimental procedure

The following instruments were used: UV and IR spectrum were measured with a Varian Conc. 100 instruments and a Perkin Elmer Spectrum One FTIR spectrometer, respectively. The ^{1}H and ^{13}C NMR spectrum were recorded with JEOL Model ECP400 Spectrophotometer [400 MHz (^{1}H) and 100 MHz (^{13}C)]. The following adsorbents were used for purification: vacuum liquid chromatography (Si-gel 60, Merck catalog number: 1.07747) and flash column chromatography (Si-gel 60 GF_{254}, Merck catalog number: 1.07749), and TLC analysis (Merck, Kieselgel 60 F_{254} 0.25 mm). Solvents used in this research are analytical grade and technical grade that were distilled before used.

Plant material

The bark of *D. confertus* was collected from Bukit Bengkirai, East Kalimantan, Indonesia, and a voucher specimen has been deposited at the Herbarium Bogoriensis, Research Center for Biology, Indonesian Institute of Science, Bogor, Indonesia (collection number SR-027).

Extraction and isolation

The dried powder of the bark of *D. confertus* Sloot (4.3 kg) was macerated with methanol (3x10 L), and evaporated under reduced pressure to give a dark brown residue (467 g). The dried methanol extract was dissolved in a small volume of MeOH (±300 mL), and added with diethyl ether to a volume ±2 L to give MeOH-diethyl ether soluble fraction (284 g) after decantation and evaporation, and an insoluble fraction (183 g). A Part of the soluble fraction (60 g) was subjected to vacuum liquid chromatography (VLC), and eluted with mixtures of *n*-hexane/EtOAc 40% to 100%, and EtOAc/MeOH 10%, 20% and 100% to give nine major fractions (F_1-F_9). Furthermore, refractionation of fraction F_3 (500 mg) by using flash column chromatography (eluent, *n*-Hexane:EtOAc 8:2), and then recrystallization of fraction F_{32} and F_{36} from this steps yielded cinnamic acid (**3**) (113 mg) and β-sitosterol (**1**) (105 m g), respectively. Fractions F_4 and F_5 performed the similar crystal form. Recrystallization and washing of this fraction with acetone and methanol yielded betulinic acid (**2**) (190 mg). Fraction F_8 (670 mg) was purified using flash column chromatography with the combination of *n*-Hexane:EtOAc 8.5:1.5 as solvent system gave α-viniferin (**4**) (210 mg).

β-sitosterol (**1**): white amorphous powder; ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 5.39 (1H, *m*, H-6), 3.51 (1H, *m*, H-3), 1.05 (3H, *s*, Me-19), 0.97 (3H, *d, J*= 6.5 Hz, Me-21), 0.90 (3H, *t, J*=7.4 Hz, Me-29), 0.87 (3H, *d, J*=6.7 Hz, Me-26), 0.85 (3H, *d, J*=6.7 Hz, Me-27) and 0.72 (3H, s, Me-18).

Betulinic acid (**2**): white powder, ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.10 (1H, *dd*, *J*= 5.48, 11.36 Hz, H-3), 2.99 (1H, *td*, *J*=4.76, 10.64 Hz, H-19), 0.91 (3H, *s*, H-23), 0.71 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.93 (3H, *s*, H-26), 0.97 (3H, *s*, H-27), 4.67 (1H, *d*, *J*= 2.2 Hz, H-29a), 4.55 (1H, *d*, *J*= 2.2 Hz, H-29b) and 1.66 (3H, *s*, H-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 40.25 (C-1), 28.21 (C-2), 79.84 (C-3), 40.14 (C-4), 57.05 (C-5), 19.54 (C-6), 35.77 (C-7), 42.10 (C-8), 52.19 (C-9), 38.38 (C-10), 22.27 (C-11), 27.08 (C-12), 39.82 (C-13), 43.76 (C-14), 31.04 (C-15), 33.59 (C-16), 57.74 (C-17), 50.63 (C-18), 48.55 (C-19), 152.24 (C-20), 31.91 (C-21), 38.51 (C-22), 28.79 (C-23), 16.30 (C-24), 16.91 (C-25), 16.84 (C-26), 15.30 (C-27), 180.40 (C-28), 110.32 (C-29) and 19.63 (C-30).

Cinnamic acid (**3**): white powder, ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 11.01 (1H, *brs*, OH), 7.81 (1H, *d, J*= 16 Hz, H-7), 7.57 (2H, *m*, H-2/6), 7.42 (3H, *m*, H-3/4/5), 6.47 (1H, d, *J*= 16 Hz, H-8). ^{13}C NMR (100 MHz, $CDCl_3$); δ_C 172.06 (C=O), 147.11 (C-7), 133.99 (C-1), 130.77 (C-4), 128.96 (C-3, C-5), 128.37 (C-2, C-6) and 117.17 (C-8).

(-)-α-viniferin (**4**): yellow powder, ^{1}H NMR (400 MHz, acetone–d_6); δ_H 7.01 (2H, d, *J*= 8.8 Hz, H-2a/6a), 6.70 (2H, *d*, *J*= 8.8 Hz, H-3a/5a,), 6.07 (1H, *brs*, H-7a), 3.95 (1H, *brs*, H-8a), 6.22 (1H, *d*, *J*= 1.8 Hz, H-12a), 6.71 (1H, *d*, *J*= 1.8 Hz, H-14a), 7.02 (2H, *d*, *J*= 8.8 Hz, H-2b/6b), 6.78 (2H, *d*, *J*= 8.8 Hz, H-3b/5b), 4.90 (1H, *d*, *J*= 6.2 Hz, H-7b), 4.60 (1H, *d*, *J*= 6.2 Hz, H-8b), 6.24 (1H, *d*, *J*= 2.0 Hz, H-12b), 5.98 (1H, *d*, *J*= 2.0 Hz, H-14b), 7.22 (2H, *d*, *J*= 8.8 Hz, H-2c/6c), 6.76 (2H, *d*, *J*= 8.8 Hz, H-3c/5c), 5.92 (1H, *d*, *J*= 10.0 Hz, H-7c), 4.68 (1H, *d*, *J*= 10.0 Hz, H-8c), 6.20 (1H, *d*, *J*= 2.0 Hz, H-12c) and 6.59 (1H, *d*, *J*= 2.0 Hz, H-14c). ^{13}C-NMR (100 MHz, acetone–d_6); δ_C 132.0 (C-1a), 128.2 (C-2a/6a), 115.7 (C-3a/5a), 158.2 (C-4a), 86.4 (C-7a), 46.4 (C-8a), 141.2 (C-9a), 120.9 (C-10a), 160.6 (C-11a), 98.0 (C-12a), 159.3 (C-13a), 106.2 (C-14a), 132.5 (C-1b), 128.7 (C-2b/6b), 116.1 (C-3b/5b), 158.4 (C-4b), 95.6 (C-7b), 55.7 (C-8b), 141.2 (C-9b), 118.8 (C-10b), 161.6 (C-11b), 98.0 (C-12b), 159.3 (C-13b), 108.6 (C-14b), 132.0 (C-1c), 128.1 (C-2c/6c), 116.1 (C-3c/5c), 157.9 (C-4c), 90.0 (C-7c), 52.8 (C-8c), 138.7 (C-9c), 119.7 (C-10c), 161.7 (C-11c), 96.9 (C-12c), 160.7 (C-13c) and 105.8 (C-14c).

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Cytotoxic Activity

The cytotoxicity was determined by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay against P388 and Vero cell lines. The assay was performed as described by Mosmann (1983)[17], but with slight modifications. MTT was first prepared are a stock solution of 5 mg/ml in phosphate buffer (PBS, pH 7.2) and filtered. Samples of different concentration of the extracts were prepared in triplicates. At the end of the treatment period (72h), 20 □ l of MTT solution was added to each sample and incubated for 3 hours. After 72 h, supernatants were discarded and 50 µl of MTT stock solution (5 mg/ml) were added to each well and the plates were further incubated for four hours. After that, supernatant was removed, and the formazan crystals were dissolved with 100 µl DMSO. The amount of MTT-formazan was directly proportional to the number of living cells, and was determined by measuring the optical density (OD) at 570 nm using microplate reader (□ Quant Universal Microplate Spectrophotometer, BIOTEK Instrument, Inc.). The IC_{50} values represented the concentration that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells [18].

RESULTS AND DISCUSSION

The methanol extract of the stem bark of *D. confertus* was separated by combination of vacuum liquid and flash column chromatographies have resulted the isolation and characterization of compounds **1**-**4**. The structure of these compounds were identified on the basis of spectroscopic data evidence as steroid; β-sitosterol (**1**)[19], pentacyclic triterpene; betulinic acid (**2**)[20], cinnamic acid (**3**) [21], and oligomeric resveratrol; α-viniferin (**4**)[12] (Fig. 1). All compounds were isolated for the first time from this plant.

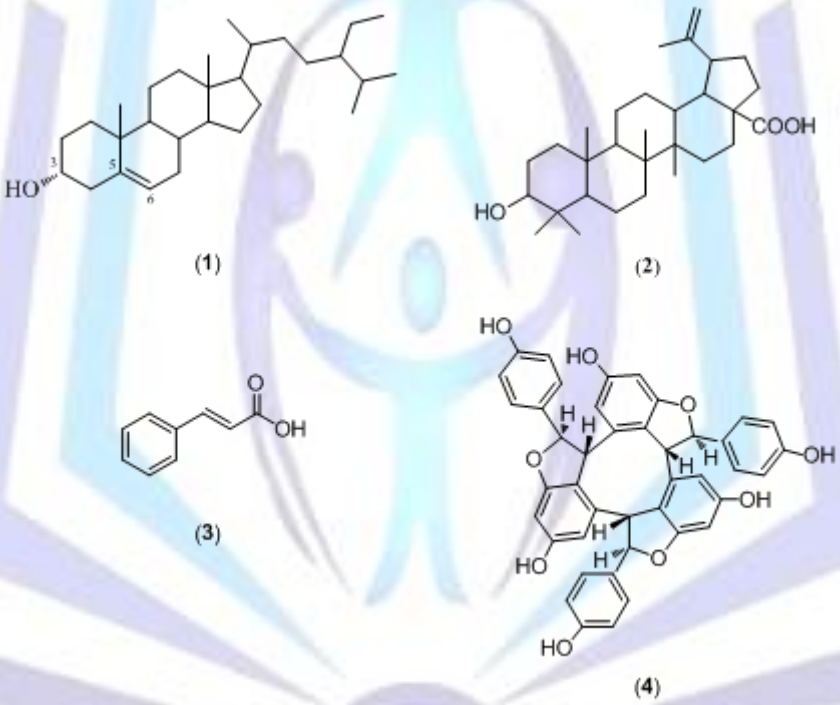

Fig. 1. The structure of isolated compounds

The cytotoxicity of all isolated compounds from the bark of *D. confertus* was tested against P-388 mice leukemia and Vero normal kidney monkey cell lines. From the assay, cinnamic acid (**3**) and betulinic acid (**2**) demonstrated potent activity against P-388 cell lines with IC_{50} value of 2.25 and 5.10 µg/ml, respectively. However, they showed different response against normal vero cell, in which betulinic acid (**2**) was toxic to the cell (IC_{50} 6.19 µg/ml), while cinnamic acid (**3**) was not toxic (IC_{50} 69823.24 µg/ml). On the other hand, other compounds were only in the range of the lowest and not active against both cell lines (Table 1). This fact indicated that only **3** displays high potency as anticancer agent, as it is only toxic to the cancer cell and not to the host cell.

Table 1. Inhibitory concentration of 50% by P-388 and Vero cell lines

Compound	Cytotoxic activity (IC_{50} µg/ml)	
	P-388	Vero
β-Sitosterol (1)	nd	nd
Betulinic acid (2)	5.10	69823.24
Cinnamic acid (3)	2.25	6.19
α-Viniferin (4)	71.00	438.26

nd = not detected

In this study, compound **3** is more active compared to **2** even though they contain similar acid group. This may be due to the existence of conjugate system in the skeleton of **3**, which activated its aromatic ring. This suggestion was supported by Otero et al. (2013) [22], which found that the cytotoxicity of chalcone and coumarin increased with the presence of conjugated double bond in the side chain. The similar case was displayed by compound **2**, which showed stronger activity compared to **1**. Several literatures reported that compound **2** is a pentacyclic triterpenoid that commonly studied, and the studies have been focused on the modifications of carbons C-3 and C-28. Ding et al. (2013) [23] reported that electron-donating groups at the C-28 and C-3 of **2** would improve the activity.

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REFERENCES

[1] Ashton, P.S., Arnold, A., 1983. Flora Malesiana: Spermatophyta I, in: Steenis, C.G.G.J. (Ed.), Flora Malesiana. Noordhoff-Kolff, pp. 391-436.

[2] Newman, M.F., Burgess, P.F., Whitmore, T.C., 1998. Manuals of dipterocarps for foresters: Sumatra medium and heavy hardwoods: Dipterocarpus, Dryobalanops, Hopea, Shorea (balau). Center for International Forestry Research, Bogor, Indonesia.

[3] Syah, Y.M., Aminah, N.S., Hakim, E.H., Aimi, N., Kitajima, M., Takayama, H., Achmad, S.A., 2003. Two oligostilbenes, cis- and trans-diptoindonesin B, from Dryobalanops oblongifolia. Phytochemistry 63, 913-917.

[4] Muhtadi, Hakim, E.H., Juliawaty, L.D., Syah, Y.M., Achmad, S.A., Latip, J., Ghisalberti, E.L., 2006. Cytotoxic resveratrol oligomers from the tree bark of Dipterocarpus hasseltii. Fitoterapia 77, 550-555.

[5] Wibowo, A., Ahmat, N., Hamzah, A.S., Sufian, A.S., Ismail, N.H., Ahmad, R., Jaafar, F.M., Takayama, H., 2011. Malaysianol A, a new trimer resveratrol oligomer from the stem bark of Dryobalanops aromatica. Fitoterapia 82, 676-681.

[6] Wibowo, A., Ahmat, N., Hamzah, A.S., Ismail, N.H., Ahmad, R., Jaafar, F.M., 2012a. Resveratrol oligomers from the stem bark of Dryobalanops aromatica. Biochem. Syst. Ecol. 40, 62-64.

[7] Wibowo, A., Ahmat, N., Hamzah, A.S., Low, A.L.M., Mohamad, S.A.S., Khong, H.Y., Sufian, A.S., Manshoor, N., Takayama, H., 2012b. Malaysianol B, an oligostilbenoid derivative from Dryobalanops lanceolata. Fitoterapia 83, 1569-1575.

[8] Pryce, R.J., and Langcake, P., 1977. α-Viniferin: An antifungal resveratrol trimer from grapevines. Phytochemistry 16, 1452-1454.

[9] Bokel, M., Diyasena, M.N.C., Gunatilaka, A.A.L., Kraus, W., Sotheeswaran, S., 1988. Canaliculatol, an antifungal resveratrol trimer from Stemonoporous canaliculatus. Phytochemistry 27, 377-380.

[10] Dai, J., Hallock, Y.F., Cardellina, J.H., Boyd, M.R., 1998. HIV-inhibitory and cytotoxic oligostilbenes from the leaves of Hopea malibatol. J. Nat. Prod. 61, 351-353.

[11] Ito, T., Akao, Y., Yi, H., Ohguchi, K., Matsumoto, K., Tanaka, T., Iinuma, M., Nozawa, Y., 2003. Antitumor effect of resveratrol oligomers against human cancer cell lines and the molecular mechanism of apoptosis induced by vaticanol C. Carcinogenesis 24, 1489-1497.

[12] Kitanaka, S., Ikezawa, T., Yasukawa, K., Yamanouchi, S., Takido, M., Kil Sung, H., Hyuk Kim, I., 1990. (+)-α-viniferin, an anti-inflammatory compound from Caragana chamlagu root. Chem. Pharm. Bull. 38, 432-435.

[13] Huang, K.S., Lin, M., Cheng, G.F., 2001. Anti-inflammatory tetramers of resveratrol from the roots of Vitis amurensis and the conformations of the seven-membered ring in some oligostilbenes. Phytochemistry 58, 357-362.

[14] Gunawardena, Y.A.G.P., Sotheeswaran, S., Sultanbawa, M.U.S., Surendrakumar, S., Bladon, P., 1986. Another antibacterial polyphenol, copalliferol B, from Vateria copallifera (Dipterocarpaceae). Phytochemistry 25, 1498-1500.

[15] Zgoda-Pols, J.R., Freyer, A.J., Killmer, L.B., Porter, J.R., 1996. Antimicrobial Resveratrol Tetramers from the Stem Bark of Vatica oblongifolia ssp. oblongifolia. J. Nat. Prod. 65, 1554-1559.

[16] Muhtadi, 2007. Phytochemical Investigation of Some Indonesian Dipterocarpaceae Species of the Genus Dipterocarpus, Doctoral Program, Postgraduate School. ITB, Bandung, p. 142.

[17] Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55-63.

[18] Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., Boyd, M.R., 1988. Feasibility of Drug Screening with Panels of Human Tumor Cells Lines Using a Microculture Tetrazolilum Assay, Cancer Res., 48, 589-601.

[19] Lee, I.K., Kim, M.A., Lee, S.Y., Hong, J.K., Lee, J.H., Lee, K.R., 2008. Phytochemical constituents of Schizonepeta tenuifolia Briquet. Nat. Prod. Sci. 14, 100-106.

[20] Barba, B., Díaz, J.G., Herz, W., 1992. Anthraquinones and other constituents of two Senna species. Phytochemistry 31, 4374-4375.

[21] Bourne, E.J., MacLeod, N.J., Pridham, J.B., 1963. The identification of naturally occurring cinnamic acid derivatives. Phytochemistry 2, 225-230.

[22] Otero, E., Robledo, S.M., Díaz, S., Carda, M., Muñoz, D., Paños, J., Vélez, I.D., Cardona, W., 2013. Synthesis and leishmanicidal activity of cinnamic acid esters: structure-activity relationship. Med. Chem. Res., 1-9.

[23] Ding, W., Sun, M., Luo, S., Xu, T., Cao, Y., Yan, X., Wang, Y., 2013. A 3D QSAR study of betulinic acid derivatives as anti-tumor agents using topomer CoMFA: Model building studies and experimental verification. Molecules 18, 10228-10241.