2012 3rd International Conference on Chemistry and Chemical Engineering
IPCBEE vol.38 (2012) © (2012) IACSIT Press, Singapore

Oligoresveratrol Isolated from Stem Bark of *Hopea Odorata* as Antioxidant and Cytotoxicity againt Human Cancer Cell Line

Sri Atun[+], Nurfina Aznam and Retno Arianingrum

Department Chemistry education, Yogyakarta State University, Karangmalang, Depok, Sleman, Yogyakarta, 55281, Indonesia

Abstract. From the stem bark of *Hopea odorata*, four known resveratrol derivatives, named balanocarpol (1), hopeaphenol (2), ampelopsin H (3), and hemlesyanol C (4) were isolated. The structure was elucidated by NMR spectroscopy, including 1D and 2D NMR. Some compounds showed as antioxidant activity and cytotoxicity againt Hela-S3 and Raji cell lines.

Keywords: *Oligoresveratrol, Hopea odorata, Antioxidant, Cytotoxicity*

1. Introduction

Hopea is one the main genus of Dipterocarpaceae, consisting of approximately 100 species and widely distributed in Indonesia specially in Kalimantan [1,2] and until now only few species have been investigated. This family of plant is known to produce a variety of resveratrol oligomers [3-9]. These structures are very interesting and showed interesting biological activity, such as antibacterial, anticancer, antihepatotoxic, and anti-HIV [3-9]. Thus Dipterocarpaceae plants are very promising for chemical research in natural product and pharmaceutical industry. In our continuing phytochemical study of the Dipterocarpaceae family occuring in Indonesia, we have examined resveratrol oligomer constituents from some species of *Hopea odorata. Hopea* is widely distributed in tropical rain forest of Sumatra, Malaysia, up to the Andaman islands, and it is locally known as " merawan hitam" or " pengarawan" [2]. This paper will report our first investigation of four resveratrol derivatives from the stem bark of these species. The structure of these compounds were derived based on the analysis of the UV, IR, MS and NMR including 1D and 2D NMR (^{1}H- ^{1}H COSY, HMQC, HMBC and NOESY) spectra.

2. Materials and Methods

2.1. Plant Material

Samples of the stem bark of *H. odorata* was collected in December 2003 from the Experimental Garden in Carita, Banten, Indonesia. The plant was identified by the staff at the Herbarium Bogoriense, Kebun Raya Bogor, Bogor, and a voucher specimen had been deposited at the Herbarium.

2.2. Extraction and Isolation

From the dried and milled stem bark of *H. odorata* (3.8 kg) was extracted exhaustively with acetone. The acetone extract on removal of the solvent under reduced pressure gave a brown residue (450 g). A portion (40 g) of the total acetone extract was fractionated by vacuum liquid chromatography (VLC) and purified by repeated column chromatography on silica gel eluted with various solvent systems was isolated four componds, namely balanocarpol (**1**) (300 mg), hopeaphenol (**2**) (1500 mg), ampelopsin H (**3**) (250 mg), and hemlesyanol C (**4**) (120 mg).

[+] Corresponding author. Tel.: +62-0274-586168 ex 218; fax: + 62-0274-548203
E-mail address: Atun_1210@yahoo.com

2.3. Spectroscophy and Chromatography Analysis

UV and IR spectra were measured with Varian Cary 100 Conc and Shimadzu 8300 FTIR, respectively. ^{1}H and ^{13}C NMR spectra were recorded with Jeol JNM A-5000 spectrometers, operating at 600.0 MHz (^{1}H) and 150.0 MHz (^{13}C) using residual and deuterated solvent peaks as internal standards. MS spectra were obtained with a JMS-AM 20 spectrometer, using the mode FAB. Vacuum liquid chromatography (VLC) was carried out using Si gel Merck 60 GF_{254} (230-400 mesh), column chromatography using Si-gel Merck 60 (200-400 mesh), and TLC analysis on precoated Si gel plates Si-gel Merck Kieselgel 60 F_{254} 0.25 mm, 20 x 20 cm.

2.4. Antioxidant test

Activity test as antioxidant conducted by radical scavenger activity from chloroform and ethyl acetate with Halliwel method[10]. In reaction tube adding 0.1 ml sollution deoksiribosa 3mM; 0.01 mL sample at various concentration; 0.1 mL ascorbat acid; 0.1 mL hydrogen peroxide 0.1mM, and 0.59 mL sollution buffer phosphate pH 7.4 then homogenized. Reaction by the start of addition of iron (II) sulphate sollution. Mixture that solution and incubation for 30 minute in temperature 37 ^{o}C. The same thing is also conducted at blank that contain reagen in common but not contain compound analysed. Reaction is stoped with addition 3 mL tiobarbiturat acid. Then heated for 15 minute in temperature 80 ^{o}C. The colour of that solution formed measured its absorption at maximum wavelength 532 nm. We calculated of hydroxyl radical scavenger percentage as follows :

$$\% \text{ hydroxyl radical scavenger} = \frac{Atp - Ap}{Atp} \times 100\ \%$$

Atp = absorption without sampel; Ap = absorption with sampel

2.5. In-vitro cytotoxicity test

The in-vitro cytotoxicity test was investigated using 96 wells plate with cell density 2x104 cells per ml. Into each well was added with 100 µl cells in culture medium (87.5% RPMI 10.4 g/L; 2% penstrep; and 10% FBS) and was then incubated in CO_2 incubator for 12-24 hours at 370C. Each sample was dissolved in culture medium containing 0.05% DMSO, and 100 µl of each sample in the different concentrations was added into each well in triplicate and was then incubated in CO_2 incubator for 12- 24 hours at 37^{o}C. MTT solution (10 µl per 100 µl medium) was added to all wells of an assay, and plates were incubated for 4 hours at 37^{o}C in CO_2 incubator. As much as 100µl formazon (10% SDS and 0. 01 N chloride acid) was added into each well and mixed on a shaker for 5 minutes. The wells were incubated in the dark room for 12- 24 hours at room temperature. The absorbance was measured using multiwell scanning spectrophotometers (ELISA reader) at wavelength 595 nm. The absorbance is directly proportional to the number of living cells. So the dead cell could be calculated to determine LC_{50}.[11]

3. Results and Discussion

Balanocarpol (1) was obtained as a pale yellow powder, m.p. 230 ^{o}C, UV (MeOH) $\lambda_{max.}$ (log ε) : 227 (5.6); 283 (3.76) nm, IR (KBr) $\upsilon_{max.}$: 3384; 1608; 1405; 1350; 1240; 1132; 1037; 995; 833 cm^{-1}, ^{1}H and ^{13}C NMR (Me_2CO-d_6, 600.0 and 150 MHz). FABMS *m/z* 470 [M^{+}] ($C_{28}H_{22}O_7$). Its UV spectrum showed absorption maximum at 283 nm suggesting the presence of unconjugated phenolic chromophore. The IR spectrum exhibited hydroxyl group (3384 cm^{-1}), C=C aromatic (1608; 1405; 1350 cm^{-1}), and monosubtituted benzene (833 cm-1), these spectral characteristic absorptions supporting **1** to be an oligoresveratrol. The positive ion FABMS exhibited an [M]$^{+}$ ion at *m/z* 470 consistent with a molecular formula $C_{28}H_{22}O_7$ for a resveratrol dimer and this suggestion was supported by the NMR data. ^{13}C NMR spectra showed six signals for oxyaryl carbon at δ 159.2 (C-4a), 157.4 (C-11a), 156.9 (C-13a), 155.8 (C-4b), 159.2 (C-11b), and 159.7 (C-13b) ppm, characteristics for resveratrol dimer. Additionally, the ^{13}C NMR also exhibited one oxyalkyl carbon at δ 73.2 (C-8b), indicating that C-8b was attached to a hydroxyl fungtional group. The ^{1}H NMR spectrum of **1** in acetone-d_6 exhibited signals for two sets of 4-hydroxybenzene at δ 7.48 (*d*, *J* = 8.8 Hz) and 6.95 (*d*, *J* = 8.8 Hz) ppm, each 2H (ring A1) and at δ 6.75 (*d*, *J* = 9.5 Hz) and 6.42 (*d*, *J* = 9.5 Hz) ppm, each 2H (ring B1). The ^{1}H NMR spectrum also showed two sets of meta-coupled aromatic protons signals at δ 6.09 (*d*, *J* = 2.2 Hz) and 5.96 (*d*, *J* = 2.2 Hz) ppm, each 1H (ring A2), and at δ 6.20 (*d*, *J* = 2.2 Hz) and 6.25

(*d*, *J* = 2.2 Hz) ppm, each 1H (ring B2). Additionally, the ^{1}H NMR spectrum exhibited signals for a set of aliphatics proton at δ 5.70 (*d*, *J* = 9.5 Hz) and 5.16 (*d*, *J* = 9.5 Hz), each 1H, characteristic for *trans*-2,3-diaryl-dihydrobenzofuran moiety, and signals assignable two coupled aliphatic protons at δ 4.89 (*br s*) and 5.39 (*br s*) ppm, each 1H. These spectral data indicated that compound **1** has a dimeric stilbene skeleton as part of its structure.

Hopeaphenol **(2)** was obtained as a pale white powder, m.p. 230 ^{o}C, UV (MeOH) $\lambda_{max.}$ (log ε) : 227) 206 (6,31); 227 (sh) (6,02); 283 (5,33) nm, IR (KBr) $\upsilon_{max.}$: 3400; 1615; 1456; 1350; 1240; 1132; 1037; 995; 833 cm^{-1}, and TLC compared with authentic sample.

Ampelopsin H **(3)** was obtained as a pale yellow powder, m.p. 240 ^{o}C, UV (MeOH) $\lambda_{max.}$ (log ε) : 225 (6.01); 230 (sh 4.83); 282 (3.65) nm, IR (KBr) $\upsilon_{max.}$: 3352; 1606; 1512; 1450; 1234; 1141; 1068; 954; 835 cm^{-1}, ^{1}H and ^{13}C NMR (Me_2CO-d_6, 600.0 and 150 MHz). FABMS *m/z* 906 $[M+H]^+$ ($C_{56}H_{42}O_{12}$). Ampelopsin H (**3**) with absorption maxima observed at 282 nm in the UV spectrum attributable to the phenol rings. The IR spectrum exhibited hydroxyl group (3352 cm^{-1}), C=C aromatic (1606-1512 cm^{-1}), and monosubtituted benzene (835 cm^{-1}). Its molecular formula of $C_{56}H_{42}O_{12}$ was established by FABMS, showing a $[M+H]^+$ ion at m/z 906, which together with the NMR spectral data, suggested that **3** was a resveratrol tetramer. The NMR data (^{1}H and ^{13}C), however showed number of signal corresponding to half the molecular formula, so was suggested that compound was **3** composed of two symmetrical structural units, and each unit was resveratrol dimmer (Table 2). The ^{1}H NMR spectrum of **3** in acetone-d_6 exhibited signals for two sets of 4-hydroxybenzene at δ 7.11 (2H, *d*, *J* = 8.4 Hz) and 6.74 (2H, *d*, *J* = 8.4 Hz) ppm, with δ 6.73 (2H, *d*, *J* = 8.4 Hz) and 6.56 (2H, *d*, *J* = 8.4 Hz) ppm. The ^{1}H NMR spectrum also showed two sets of meta-coupled aromatic protons signals at δ 6.32 (1H, *t, J* = 2.1; 2.1 Hz) ppm and 6.29 (2H, *br s*) ppm indicating the presence of a 3,5-hydroxyphenyl group. Furthermore, the aromatic proton signal at 6,21 (1H, *s*) ppm showed existence of a pentasubstituted benzena ring. Two proton signals at δ5.31 (1H, *d, J* = 2.0 Hz) ppm and d 4.33 (1H, *d, J* = 2.0 Hz) ppm showed existence of a *trans*-dihydrobenzofuran ring. Two proton signals at δ 4.29 (s) ppm and 3.85 (s) ppm indicated that both protons were at different locations.

Hemlesyanol C **(4)** was obtained as white brown powder, UV (MeOH) $\lambda_{max.}$ (log ε) 203 (5.31); 283 (4.33), IR (KBr) υ_{max} 3200, 1612–1454 , and 833 cm^{-1}^{1}H and ^{13}C NMR (Me_2CO-d_6, 600.0 and 150 MHz). FABMS *m/z* 906 $[M^+]$ ($C_{56}H_{42}O_{12}$). Hemlesyanol C (**4**), with absorption band (283 nm) in the UV spectrum showing the presence of aromatic rings. The IR spectrum exhibited hydroxyl group (3200 cm^{-1}), C=C aromatic (1612–1454 cm^{-1}), and monosubstituted benzene (833 cm^{-1}). The $[M^+]$ ion peak at *m/z* 906, corresponded to the molecular formula $C_{56}H_{42}O_{12}$. The ^{1}H-NMR spectrum (Table 2), showed the signals assignable to four 4-hydroxyphenyl groups at δ 7.58 (2H, *d*, *J* = 8.4); 6.91 (2H, *d*, *J* =8.4 Hz); 6.11 (2H, *d*, *J* = 8.4 Hz); 6.40 (2H, *d*, *J* = 8.4 Hz); δ 5.77 (2H, *d*, *J* = 8.8 Hz); 6.20 (2H, *d*, *J* = 8.8 Hz); 7.07 (2H, *d*, *J* = 8.4 Hz); 6.85 (2H, *d*, *J* = 8.4 Hz). The presence of a 3,5-dihydroxyphenyl group at δ 5.91 (2H, *d*, *J* = 2.5 Hz) H-10d and 14d, δ 6.11 (*d*, *J* = 2.5 Hz) H-12d, and two sets of meta coupled aromatic protons on 1,2,3,5-tetrasubstituted benzene rings at δ 6.23 (*d, J* = 2.2 Hz), H-12a; 6.05 (*d*, *J* = 2.2 Hz), H-14a; 6.29 (*d*, *J* = 2.7 Hz), H-12c and 5.91 (*d*, *J* = 2.7 Hz), H-14c were also exhibited. The spectrum further showed the signals due to an aromatic proton on a pentasubstituted benzene ring at δ 6.00 *(s*), H-12b, a sequence of four aliphatic methine protons coupled successively in the COSY spectrum in the order δ 4.40 (*d*, *J* = 3.3 Hz), H-7b; 4.16 (*t*, *J* = 3.3; 3.3 Hz), H-8b; 3.88 (*d*, *J* = 5.8 Hz), H-7c and 3.19 (*d*, *J* = 5.8 Hz), H-8c, and two sets of mutually coupled aliphatic protons δ 5.68 (*d*, *J* = 10.6 Hz), H-7a and 5.35 (*d*, *J* = 10.6 Hz), H-8a; δ 5.08 (*d*, *J* = 3.3 Hz), H-7d and 3.65 (*d*, *J* = 3.3 Hz), H-8d, in addition to ten phenolic hydroxyl groups (δ 6.46 -8.57) ppm. These results suggested that compound was a stilbene composed of four resveratrol units. Analysis of the HMQC and HMBC spectra enabled the complete assignments of all protonated carbons and quarternary carbons corresponding to respective resveratrol units (A-D). The HMBC spectrum showed cross peaks indicating long range correlations between H-7b/ C-14b, C-8c, H-8b/ C-14b, and C-9c, H-7c/ C-14b; H-8c/ C-14b; and C-8b. Therefore, it may be concluded that the **4** is hemlesyanol C, a resveratrol tetramer, isolated from *Shorea hemsleyana* for the first time [6]. The structure of four oligoresveratrol can shown at Fig. 1.

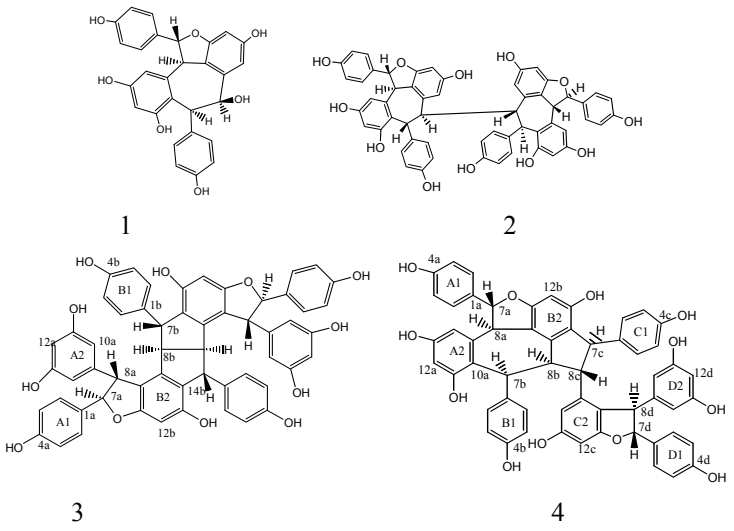

Fig. 1: Structure four oligoresveratrol compounds isolated from *H.odorata*

Activity test as antioxidants based on radical scavenger activity using the Halliwel method [10], is shown at Table 1. The data IC_{50} showed that the activity as radical hydroxyl scavenger from hopeaphenol (2) was more active than ascorbic acid and the IC_{50} of oligoresveratrol, balanocarpol (1) and ampelopsin H (3) showed them to be less active. For oligoresveratrol that activity as hydroxyl radical scavenger was due to the existence of phenol ring, stability of molecular structure, and existence of double bonds of olefinic unit. Phenol ring can trap hydroxyl radical by releasing hydrogen radical, that by condensation with hydroxyl radical, form water molecules, whereas radical phenol will be stabilized by resonance. That is, resveratrol compound is referred for development as antioxidant. An antioxidant is substance that can prevent or slow down the reactions of radical oxidation. The role antioxidant in body is to reduce the amount free radicals, like ROS (reactive oxygen species) that can be formed in course of metabolism in organism. Antioxidant also can function to protect low density lipoprotein (LDL) from oxidation reaction, thus preventing the occurrence of arteriosclerosis.

The cytotoxic activity of the samples against HeLa-S3 and Raji cell measured as LC_{50} were provided in Table 1. HeLa-S3, a *continuous cell line* that living as adherent cell, is a cell derivate of ephythell cell of human cervix cancer[12]. Table 1 show that the highest cytotoxic activity against HeLa-S3 and Raji cell is ampelopsin H. This compound is more active than doxorubicin. Doxorubicin, a medicine for lymphoma, leukaemia and acute tumor, was also measured its cytotoxic activity as standard comparison. It is necessary to carry out further investigation about the relationship between the structure and the activities of these compounds. Some studies of curcumin that has been known as anticancer indicated that the existence of hydroxyl group at ortho position and β-dicarbonil gave a big contribution as inducer of enzymes in phase two that their function as protector from carcinogenesis as epoxy hydrolyse, glutathione S-transferase (GST), and NAD(P)H quinone reductase (QR)[13].

Until now there is no information about ampelopsin H activity can repress E6 and E7 expression. Based on some anticancer compound like curcumin[13], it is possible that the antiproliferative activity of ampelopsin H (3) is initiated by bounding between this compound and protein. Therefore it cause the protein was recognize by proteosome that it degrade this protein. The anticancer activity on Raji cell related with the capability of anticancer to inhibit c-*myc, Bcl-2*, *p-53* mutant, and the capability to increase Fas expression. This research show that ampelopsin H (3) have ability to inhibit proliferation of HeLa-S3 and Raji cell. The inhibiting is possible occur at cell cycle progression by inhibit protoonkogen like *c-myc*, and also able to activate tumor suppressor like p53, *pRb* able to inactive *Bcl-2* [14].The potent ability to induce apoptosis was shown in these compounds and it was also suggested that ampelopsin H (3) is a possible candidate as a chemopreventive and chemotherapeutic agent. However, further experiments should be required to assess the anticancer effect of ampelopsin H (3) in animal model and also to define the mechanisms of this compounds at the molecular level, which are under current progress in our laboratory.

Table 1: Data of activity test as radical scavengers and cytotoxicity against HeLa-S3 and Raji cell

	Activity test as radical scavengers IC_{50} (µg/ml)	Cytotoxicity against HeLa-S3(LC_{50} µg/ml)	Cytotoxicity against Raji cell (LC_{50} µg/ml)
Balanocarpol (1)	1802.3	682.16	235.29
Hopheaphenol (2)	61.8	1931.52	781.49
Ampelopsin H (3)	4840.0	129.72	34.69
Hemlesyanol C (4)	425.5	557.44	292.15
Ascorbic acid	83.9	96.82	94.38
Butylated Hydroxy Toluene (BHT)	1328.1	-	-

4. Conclusion

In this paper we concluded that oligoresveratrol isolated from the stem bark of *Hopea odorata* consist of dimer and tetramer resveratrol. Some compounds have biological activity as antioxidant and cytotoxic effect against Raji and HeLa-S3 lines cell. Hopeaphenol (2) showed the highest activity as antioxidant, whereas ampelopsin H (3) gave the highest cytotoxic effect against HeLa-S3 and Raji cell.

5. Acknowledgment

This work has been supported by Fundamental Research grant from DIKTI (2007-2008). The authors are grateful to the experimental Garden in Carita, Pandeglang, Banten, Indonesia and Herbarium Bogoriensis for gift of the sample and identification of the plant specimen.

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