

Isolation of an Anti-Cancer Asperuloside from *Hedyotis corymbosa* L.

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ABSTRACT

Hedyotis corymbosa L., with local name rumpit mutiara, is an anti-inflammatory, anti-cancer and hepatoprotective traditional medicine. The ethanol extract of *H. corymbosa* L. shows inhibitory activity to human YMB-1 breast cancer cell line with an IC_{50} of 6.51 $\mu\text{g/mL}$. The methylene chloride fraction shows a potential cytotoxic activity with an IC_{50} of 2.75 $\mu\text{g/mL}$. To obtain a lead compound, the extract was further purified by column chromatography. A pure compound is obtained which shows inhibitory activities against YMB-1, HL60 and KB human cell lines with IC_{50} values of 0.7; 11.0 and 104.2 $\mu\text{g/mL}$, respectively. Based on the 1D and 2D FT-NMR data, the isolated compound is an asperuloside.

Keywords: asperuloside, *Hedyotis corymbosa*, HL60, KB, leaf, Rubiaceae, YMB-1

INTRODUCTION

Hedyotis corymbosa L., with local name rumpit mutiara, is one of the herbal medicines used by Indonesian people to treat diseases or to maintain their health [1]. It is also being used to treat inflammation, hepatitis, cancer. There are a wide variety of products from *H. corymbosa* that have been sold for a long period of history [2].

H. corymbosa is one of the species from *Hedyotis* (genus), *Rubiaceae* (family), *Rubiales* (ordo), *Dicotyledoneae* (class), *Angiospermae* (sub-division), and *Spermatophyta* (Division). It grows well in dry and sandy soil, along rivers and coasts and in the forests [3-5]. They widely grow in Indonesia, are also found in Malaysia and India. Previous studies on some *Hedyotis* species have yielded indole alkaloids, anthraquinones, lignans, triterpenes, flavonoids as well as iridoids. the three new iridoid glycosides are identified as hedycoresides A-C [6].

Many *Hedyotis* species (*Rubiaceae*) are also used in traditional Chinese medicine (TCM) for the treatment of appendicitis, tonsillitis, hepatitis, dysentery, snake bites, and bruising [7]. The chemical constituents of

this genus include iridoid glycosides, triterpenoids, flavonoids, anthraquinones, coumarins, lignans, and alkaloids, some compounds exerting anti-inflammatory, neuroprotective, and cytotoxic effects [8]. *H. corymbosa* (Linn.) Lam. is an annual herb widely distributed in the southeast and southwest of China [5]. The whole plant is applied in the clinic against malaria, intestinal abscess, boils, scald, and some kinds of tumors, such as gastric, esophageal, and colorectal carcinomas [9-11].

Investigations on the methanol extract of *H. corymbosa* reveal various bioactivities, namely antibacterial, anti-inflammatory, free radical-scavenging, cytotoxic, and hepatoprotective [3,12]. In this investigation, we investigate the cytotoxicity of ethanol extract from the whole plant, its fractions and a lead compound of *H. corymbosa*. We use Sulforhodamine B method against YMB-1, HL60, and KB cell lines to determine the half maximal inhibitory concentration (IC_{50}). Based on the active fraction, we have isolated a cytotoxic

Table 1. Cytotoxicity of compound 1 (asperuloside) and antimycin A3

Compound	IC_{50} ($\mu\text{g/mL}$)		
	YMB-1	HL60	KB
Asperuloside (1)	0.7	11.0	104.2
Antimycin A3	0.3	1.7	2.9

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Table 2. ^1H and ^{13}C -NMR spectral for compound **1** and asperuloside

No	1 (CD_3OD , 300 MHz)		Asperuloside (CD_3OD , 400 MHz) [16]	
	δ_{H} (m, J Hz)	δ_{C}	δ_{H} (m, J Hz)	δ_{C}
1	5.92 (d, 2)	100.1	5.63 (d, 2)	99.9
2				
3	7.21 (d, 2)	150.3	7.21 (d, 2)	150.3
4		106.3		106.2
5	3.57 (m)	37.5	3.21 (m)	37.4
6	5.47 (dd, 1.5; 6.8)	86.4	5.47 (d, 6.8)	86.3
7	5.86 (d, 1.5)	129.0	5.86 (m)	128.9
8		144.1		144.3
9	3.51 (m)	45.3	3.55 (m)	45.2
10	4.58 (dd, 12.3; 7.8)	64.4	4.58 (br s)	64.4
1'	4.6 (d, 7.8)	93.4	4.96 (d, 7.5)	93.3
2'	3.6 (m)	77.9		77.9
3'	3.22 (m)	78.4		78.4
4'	3.3 (t, 7.0)	71.6		71.6
5'	3.4 (dd, 7.0; 4.8)	74.7		73.8
6'	3.82 (dd, 12; 2.1)	61.9		61.9
	3.41 (dd, 12; 3.8)			
-C=O		172.3		172.3
CH_3CO -	2.0 (s)	172.6	1.98 (s)	172.6

compound and identified its structure.

MATERIALS AND METHODS

Materials

Hedyotis corymbosa samples were collected from Indonesian Medicinal and Aromatic Crops Research Institute. Species identification of the species was performed by the Research Center for Biology, Indonesian Institute of Sciences (Cibinong, Indonesia). Whole plant samples were washed and powder dried. The crude extract was obtained using technical grade ethanol as a solvent. Extracts were also obtained with *n*-hexane, methylene chloride, and ethyl acetate as solvents.

Isolation and Identification of Methylene chloride Fraction

The entire *H. corymbosa* was used in this study. Ethanol extract was dissolved in methanol-water and fractionated in *n*-hexane, methylene chloride, and ethyl acetate. The active methylene chloride (MTC) fraction was purified by column chromatography and crystallized in dichloromethane-*n*-hexane. A pure compound was identified by one dimensional (1D) and 2D (HSQC and HMBC) FT-NMR (Bruker 300 MHz).

Cell Culturing

YMB-1 (breast cancer), HL60 and KB cell lines were obtained from Department of Material Science, Faculty of Science—Osaka City University, Osaka—Japan. The cell lines were cultivated at 37°C with 5% CO_2 in RPMI60 or DMEM (GIBCO) medium supplied

with 10% v/v Fetal Bovine Serum (Sigma), 1% antibiotic-antimycotic (GIBCO).

Assay for Cytotoxic Activity

The cytotoxic assay was determined using Sulforhodamine B (SRB) method (reference— is this provided by a kit). Cell suspensions (10^4 cells/mL) were seeding to each well and the cells were incubated for at 37°C under 5% CO_2 . After 24h, medium was replaced and extracts were added at final concentrations ranging from 100 to 3.125 $\mu\text{g}/\text{mL}$. After 24 hours, cold 50% trichloroacetic acid was added and the plates were incubated at 4°C for 30 minutes, washed with tap water 5 times, and air-dried. The wells were incubated with 100 μg of SRB dye dissolved in 1% acetic acid. After rinsing-off unbound dye with 1% acetic acid five times,

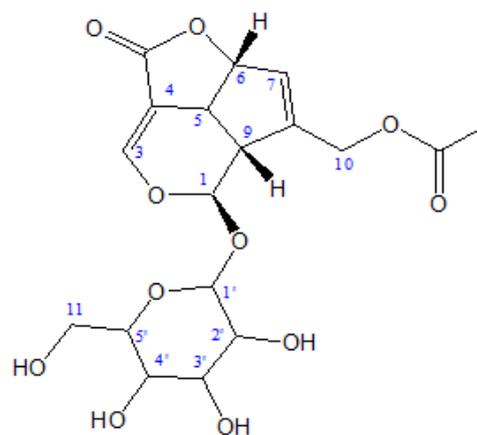


Figure 1. Structure of compound **1** (asperuloside)

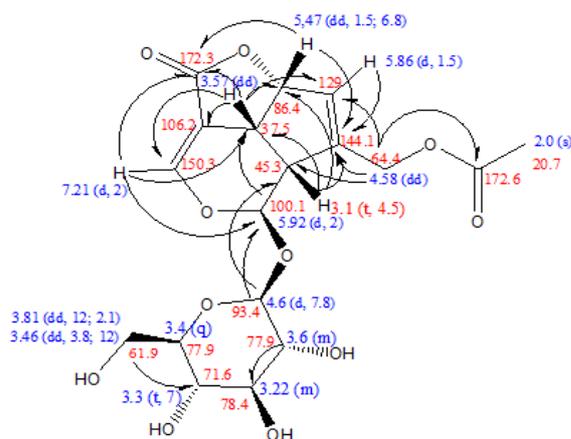


Figure 2. Summary of HMBC experiment of compound 1

the plates were air-dried. The solubilized bound dye with 200 µg/well 10 mM Tris base (pH 10) for 5 minutes on a gyratory shaker. The measurement of Optical Density (OD) at 515 nm used ELISA plate reader (Tecan Mannedorf, Switzerland). Assay was performed in triplicates per extract. Antimycin A3 was used as positive control. Concentration of samples and control is 0.2-100 µg/mL.

RESULTS AND DISCUSSION

H. corymbosa has shown cytotoxic activity to YMB-1 cell line with IC_{50} of 6.51 µg/mL. After fractionation, the methylene fraction exhibits an IC_{50} of 2.75 µg/mL. Our ethanol extract and its fraction in *n*-hexane, methylene chloride, and ethyl acetate have been previously reported to be inhibitory to breast cancer T47D cell line [13], with IC_{50} of 9.63, 33.45, 54.59 and 52.58 µg/mL, respectively.

We were able to purify the active compound that showed strong activity to inhibit both YMB-1 and HL60 with IC_{50} 0.7 and 11.0 µg/mL, respectively, and its similar to standard. The compound has no activity against KB carcinoma cell line. For control standard, we used antimycin A3, with strong cytotoxic to growth inhibition human breast cancer cell line YMB-1, human leukemia cell line HL60 and KB with IC_{50} value of 0.3, 1.7 and 2.9 µg/mL, respectively. Furthermore, it was identified by 1D and 2D NMR experiments data.

From 1H NMR spectra of compound 1 (see Table 1), there were 16 signals present. Methyl proton was found at δ_H 2.0 (s, CH_3CO-) and indicated as acetyl group substituent. There were also two methylene and ten methine groups on the spectra. The methylene group which were found at δ_H 4.58 (dd, 12.3; 7.8 Hz) and 3.82 (dd, 12; 2.1 Hz), 3.42 (dd, 12; 3.8 Hz) were

suggested to be connected with an oxygenated group and has geminal coupling. Meanwhile the methine groups at δ_H 3.2 – 3.6 suggested to be connected with $-OH$ groups, and indicated the presence of glucosyl group. It was supported by the presence of anomeric proton (H-1') at δ_H 4.60 (d, 7.8 Hz). The olefinic group that more deshielded than others that were found at δ_H 7.21 (d, 2 Hz) suggested as proton on sp^2 carbon that also linked with an oxygen atom.

Inspection of ^{13}C NMR spectra revealed about 18 signals. Methyl signal that were present at δ_C 20.7, was easily determined as carbon at δ_C 20.7. Two carbonyls, and two quaternary sp^2 carbon also present at δ_C 172.3, 172.6, 144.1 and 106.1 respectively. Four oxygenated methine carbons were signaled of δ_C 71.6 to 78.4 (non-anomeric carbon), and δ_C 93.4 (anomeric carbon). Methine carbons at δ_C 93.4 and 100.1 expected to have deshielding effect and suggested to be linked with two oxygen atom. By exposure of ^{13}C NMR, we suggested that it has monoterpene and glucopyranosyl basic skeleton. The basic skeleton of monoterpene was from ten carbon of one methylene sp^3 , two quaternary sp^2 carbon, two methine sp^3 , two methine sp^2 two oxygenated methine sp^3 , and one carbonyl (C=O). Meanwhile, $CH_3C=O$ groups suggested as substituent in monoterpene skeleton. The glucopyranosyl ring was presented by three non-anomeric carbons that also came from methine group, two anomeric carbon, and methylene groups which attached with hydroxyl group respectively. The methylene group from glucopyranosyl ring was presented by proton of δ_H 3.8 (dd); 3.41 (dd). It confirmed well in HMBC spectra by correlation of the proton with non-anomeric carbon at δ_C 71.6 (C-4'). Meanwhile, position of anomeric carbon of C-1 at δ_C 93.4, was showed by the correlation with anomeric proton at 4.6 (d) (See Table 2).

Construction of iridoid skeleton from monoterpene was started from the methine proton of δ_H 5.92 (H-1, d, 2 Hz). By using HMBC spectra, the proton of H-1 had a correlation with carbon of δ_C 45.3 (C-9) and 37.5 (C-5). Correlation between proton of H-9 at δ_H 3.1(m) with carbon of δ_C 37.5, 86.4, and 144.1 within two or three bond distance suggested that the carbons at position of C-5, C-6 and C-8 respectively. For proton of H-5 (3.57, m) which have correlation with carbon of δ_C 150.3 (C-3), 106.3 (C-4), 172.3 (C=O), 86.4 (C-6) and 129.0 (C-7) confirmed the iridoid skeleton. Correlation between methylene proton (H-10) with the carbonyl of δ_C 172.6 suggested that it adjacent to the carbonyl. So finally, we suggested the position of the

methyl of δ_{H} 2.0 (s) is also adjacent to the carbonyl of δ_{C} 172.6 due there is no other empty carbon space to be attached. The glucosyl group was located in C-1 based on the presence of long-range coupling between H-1 at δ_{H} 4.6 (d) to at δ_{C} 100.1. The detailed HMBC experiment is summarized in Figure 1. The structure above is similar and in the agreement with asperuloside as showed in Figure 2 [6,14,15].

CONCLUSIONS

The ethanol extract and methylene chloride fraction of *H. corymbosa* L. are prospective as a potential for anticancer, to growth inhibition to YMB-1 cell line with each IC_{50} value is 6.51 and 2.75 $\mu\text{g}/\text{mL}$. A lead compound asperuloside also shown high biological activity to growth inhibition against to YMB-1 and HL60 cell line with IC_{50} is 0.7 and 11.0 $\mu\text{g}/\text{mL}$.

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REFERENCES

- Sirait N (2015) Potensi rumput mutiara (*Hedyotis corymbosa*) sebagai antikanker. Warta Penelitian dan Pengembangan Tanaman Industri. 20(3): 11-13.
- Sivaprakasm SSK, Karunakaran K, Subburaya U, Kuppusamy S, Subashini TS (2014) A review on phytochemical and pharmacological profile of *Hedyotis corymbosa* Linn. Int. J. Pharm. Sci. Rev. Res. 26(1): 320-324.
- Ahmad R., Ali AM, Israf DA, Ismail NH, Saari K, Lajis NH (2004) Antioxidant, radical-scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some *Hedyotis* species. Life Sciences. 76: 1953-1964.
- Liou WC, Lin YC, Lin YM, Chen FC (1979) *N*-Benzoyl-*L*-phenylalanyl-*L*-phenylalaninol acetate from *Hedyotis corymbosa* (L.) Lam. Chemistry. (3): 72-73
- Ho TI, Chen GP, Lin, YC, Chen FA (1986) An anthraquinone from *Hedyotis diffusa*. Phytochemistry. 25(8): 1988-1989.
- Jiang W, Kuang LS, Hou AJ, Qian M, L JZ (2007) Iridoid glycosides from *Hedyotis corymbosa*. Helvetica Chimica Acta. 90(7): 1296-1301.
- Chinese Patent Application (2014) Traditional chinese medicine composition for treating stomach cancer, application and preparation. CN 103585514 A.
- Chen YH, Chang FR, Wu CC, Yen MH, Liaw CC, Huang HC, Kuo YH, Wu YC (2006) New cytotoxic 6-oxygenated 8,9-dihydrofurocoumarins, hedyotiscone A-C, from *Hedyotis biflora*. Planta Med. 72:75.
- Zhao JF, Yuan QM, Yang XD, Zhang HB, Li L (2005) Two new iridoid glycosides from *Hedyotis tenelliflora* Blume. Helv. Chim. Acta. 88: 2532-2536.
- Ahmad R, Shaari K, Lajis NH., Hamzah AS, Ismail NH, Kitajima M (2005) Anthraquinones from *Hedyotis capitellata*. Phytochemistry. 66: 1141.
- Jiangsu College of New Medicine (1977), in HA Dictionary of the Traditional Chinese Medicines I. Shanghai Scientific and Technical Publishers, Shanghai. p. 535.
- Sadasivan S, Latha PG, Sasikumar JM, Rajashekar S, Shyamal S, Shine VJ (2006) Hepatoprotective studies on *Hedyotis corymbosa* (L.) Lam. J. Ethnopharmacology. 106: 245-249.
- Andriyani R, Risdian C, Udin Z (2011) Cytotoxicity Assay from Fractions of *Hedyotis corymbosa* Extract Against Breast Cancer Cell Line T47D. Indonesian J. Cancer Chemoprevention. 2(1): 182-186.
- Lee JH, Ku CH, Baek NI, Kim SH, Park HW, Kim DK (2004) Phytochemical constituents from *Diodia teres*. Arch. Pharm. Res. 27(1): 40-43.
- Peng JN, Feng XZ, Peng XTL (1998) Iridoids from *Hedyotis hedyotide*. Phytochemistry. 47(8): 1657-1659.
- Jae HL, Chung HK, Nam IB, Sung HK, Hee WP, Dae KK (2004) Phytochemical Constituents from *Diodia teres*. Arch Pharm Res. 27(1): 40-43.