

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

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## ABSTRACT

*Hedyotis corymbosa* L., with local name rumput 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 rumput 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.  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral for compound **1** and asperuloside

No	<b>1</b> ( $\text{CD}_3\text{OD}$ , 300 MHz)		Asperuloside ( $\text{CD}_3\text{OD}$ , 400 MHz) [16]	
	$\delta_{\text{H}}$ (m, J Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (m, J Hz)	$\delta_{\text{C}}$
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
$\text{CH}_3\text{CO}$ -	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%  $\text{CO}_2$  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%  $\text{CO}_2$ . 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  $\mu\text{g}$  of SRB dye dissolved in 1% acetic acid. After rinsing-off unbound dye with 1% acetic acid five times,

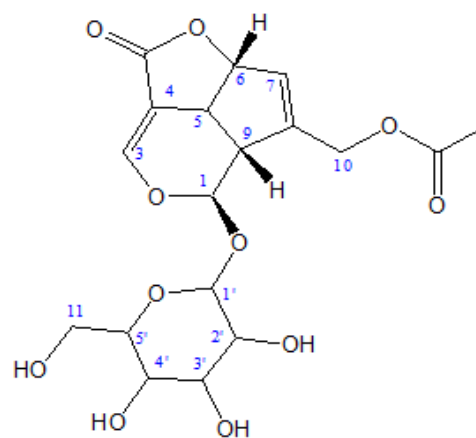


Figure 1. Structure of compound **1** (asperuloside)

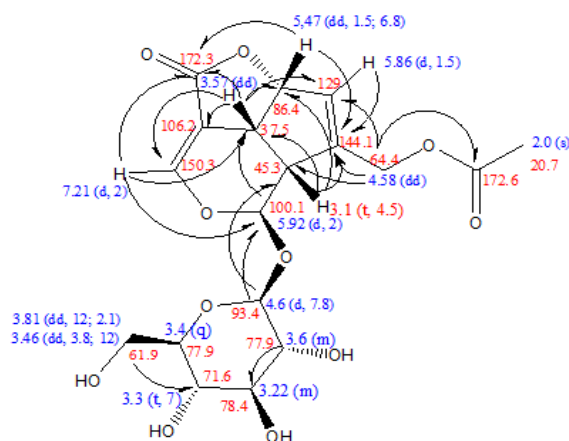


Figure 2. Summary of HMBC experiment of compound 1

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  $\delta_H$  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  $\delta_H$  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  $\delta_H$  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  $\delta_H$  4.60 (d, 7.8 Hz). The olefinic group that more deshielded than others that were found at  $\delta_H$  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  $\delta_C$  20.7, was easily determined as carbon at  $\delta_C$  20.7. Two carbonyls, and two quaternary  $sp^2$  carbon also present at  $\delta_C$  172.3, 172.6, 144.1 and 106.1 respectively. Four oxygenated methine carbons were signaled of  $\delta_C$  71.6 to 78.4 (non-anomeric carbon), and  $\delta_C$  93.4 (anomeric carbon). Methine carbons at  $\delta_C$  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  $\delta_H$  3.8 (dd); 3.41 (dd). It confirmed well in HMBC spectra by correlation of the proton with non-anomeric carbon at  $\delta_C$  71.6 (C-4'). Meanwhile, position of anomeric carbon of C-1 at  $\delta_C$  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  $\delta_H$  5.92 (H-1, d, 2 Hz). By using HMBC spectra, the proton of H-1 had a correlation with carbon of  $\delta_C$  45.3 (C-9) and 37.5 (C-5). Correlation between proton of H-9 at  $\delta_H$  3.1(m) with carbon of  $\delta_C$  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  $\delta_C$  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  $\delta_C$  172.6 suggested that it adjacent to the carbonyl. So finally, we suggested the position of the

methyl of  $\delta_{\text{H}}$  2.0 (s) is also adjacent to the carbonyl of  $\delta_{\text{C}}$  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  $\delta_{\text{H}}$  4.6 (d) to at  $\delta_{\text{C}}$  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  $\text{IC}_{50}$  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  $\text{IC}_{50}$  is 0.7 and 11.0  $\mu\text{g}/\text{mL}$ .

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