CYTOTOXIC ISOBRACTATIN (PRENYLATED XANTHONE) EPIMER MIXTURE OF *Garcinia eugenifolia*

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ABSTRACT

In search of bioactive anti-cancer compounds from natural products has been found isobractatin from n-hexane extract of Garcinia eugenifolia. Isolation was done through the stages of maceration and column chromatography. Elucidation of the structure was analyzed by spectroscopy of UV-Vis, FTIR, LC-MS, NMR proton and carbon one and two dimension data. The results found three compounds isolated from G. eugenifolia. The three compounds were stigmasterol, prenylated xanthone isobractatin and its epimer (in a mixture). The mixture of isobractatin and its epimer showed cytotoxic activity against breast cancer cell line T47D with IC_{50} = 17 µg/mL.

Keywords: *Garcinia eugenifolia; isobractatin; epimer; breast cancer; T47D cells*

ABSTRAK

Dalam penelitian senyawa bioaktif anti kanker dari bahan alam telah ditemukan isobractatin dari ekstrak n-heksana Garcinia eugenifolia. Isolasi dilakukan melalui tahap-tahapan maserasi dan kromatografi kolom. Elusidasi struktur dilakukan melalui analisa data spektroskopi UV-Vis, FTIR, LC-MS dan NMR proton, karbon satu dan dua dimensi. Hasil isolasi dari G. eugenifolia diperoleh tiga senyawa. Ketiga senyawa tersebut adalah stigmasterol, xanton terfrenilasi (adalah campuran) isobractatin dan senyawa epimernya. Senyawa isobractatin dan campuran epimernya menunjukkan aktivitas sitoksit terhadap sel kanker payudara T47D dengan IC_{50} = 17 µg/mL.

Kata Kunci: *Garcinia eugenifolia; isobractatin; epimer; kanker payudara; sel T47D*

INTRODUCTION

Cancer is a class of diseases characterized by out-of-control cell growth. Any practical solution in combating this disease is importance to public health. Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their anti-tumor actions against various cancers [1]. Breast carcinoma (BC) is commonest cancer among women and the second highest caused of the cancer death [2]. Worldwide, breast cancer accounts for 22.9% of all cancer (excluding non melanoma skin cancer) in women [3]. Most cases occur during age 45–55. It also occurs in men but is more than 100-fold less frequent than in women [4-5]. The genus Garcinia (Clusiaceae) which is encountered mainly in lowland rain forests of the tropical World has been extensively investigated from phythochemical and biological point of view. This family rich source benzophenone and xanthones [6], phrenylated xanthones, triterpenes and biflavonoids have been isolated from African and Southeast Asian properties [7].The Garcinia genus is well know to be a rich source of bioactive isophrenylated xanthones and benzophenones [8-13] Many studies have found that those compounds related on cytotoxic activity against cancer. Alpha-mangostin, a phrenylated xanthone isolated from mangosteen, has a potent cytotoxic effect against BC-1 cell. Ginkgo biloba extract contained of biflavonoids and terpenoid significantly can suppress proliferation and increase cytotoxicity in HepG2 and Hep3B [14]. Recent phytochemical investigation on stem bark of *Garcinia eugenifolia* have resulted in the isolation two compounds are rubraxanthone with have activity antimicrobial, inhibitory effects on platelet-activating factors binding and antioxidative activities, respectively and compound isocowanol is less activity [15] It is reasonably hypothesized that *G. eugenifolia* extract may contain active compounds which have cytotoxic activity against cancer cell In search for anticancer agents from natural sources, an *n*-hexane of

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Table 1. δ ^{13}C-NMR data compound **1** compared with δ ^{13}C-NMR Stigmasterol data [9]

C number	δ ^{13}C-NMR ($CDCl_3$) Stigmasterol (ppm)	δ ^{13}C-NMR of ($CDCl_3$) Compound 1 (ppm)
1	37.2	37.49
2	31.6	29.18
3	71.8	72.04
4	42,5	42.54
5	140.9	140.99
6	121.9	121.95
7	32.8	32.13
8	31.9	31.89
9	50.2	50.34
10	36.6	36.75
11	22,7	21.47
12	39.7	39.92
13	42.3	42.45
14	56.9	57.10
15	24.3	24.61
16	28.9	28.40
17	56.0	56.19
18	12.0	12.29
19	19.3	19.65
20	40.5	40.75
21	21.3	21.34
22	138.3	138.57
23	129.3	129.49
24	51.2	51.49
25	31.8	31.90
26	18,9	19.23
27	21.1	21.32
28	25.4	25.65
29	12.2	12.50

the bark of *G. eugenifolia*. The development of new therapeutic approach to breast cancer remains one of the most challenging areas in cancer research. Many tropical plants have interesting biological activities with potential therapeutic applications [3].

In this paper, we report the isolation, structure elucidation and biological evaluation on T47D cell culture of the mixture compound.

EXPERIMENTAL SECTION

Materials

Plant material of bark of *G. eugenifolia* was taken from the village Kalampangan, District Sebangau, District Palangkaraya, Central Kalimantan. The voucher specimen was identified and deposited in Herbarium Bogoriense Research Center for Biology LIPI, Cibinong Bogor Indonesia.

Instrumentation

Melting point was determined by using melting point apparatus and was uncorrected, UV spectra were measured on Hewlett-Packard (HP) 8453A, IR spectra was measures on diode array spectrophotometer FTIR Prestige-21, Shimadzu. NMR Spectra were recorded on 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR), Varian, Inova Plus, With TMS as internal standard in $CDCL_3$ as solvent. For molecule weight was measure on LC-MS, mariner Biospectrometry spectrometer using ESI system (electro ionization) and positive ion mode, Hitachi L-6200, Asquire 3000 plus-01073, Shimadzu.

Procedure

Extraction and isolation

The ground dried stem barks of *G. eugenifolia* (2.507 kg) was maceration with *n*-hexane (3 x 3 L), the *n*-hexane soluble part was evaporated under reduce pressure to give a yellow solid (23 g). This was further fractionated (20 g) by column chromatography to yield five fractions. Fraction 1 (2.14 g), fraction 2 (3.87 g) contain white crystal less soluble on MeOH further soluble compound be separated by decantation gave pure white crystal (0.125 g), fraction 3 yellow powder (4.32 g), fraction 4 (5.05 g) and fraction 5 (3.18 g). Fraction 3 subjected to column chromatography on silica gel 60 and eluted with *n*-hexane : ethyl acetate as gradient resulting yellow crystal isobractatin and epi-isobractatin (**2**, **3**) (0.754 g).

Sulphorhodamine B colorimetry for cytotoxic assay

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the anticancer activity of T47D human breast cancer cells. Cells were seeded into a 96-well plate with 3000 cells per well and incubated at 37 °C for 24 h. The cells were treated with various concentrations of the sample (0-100 µg/mL) with doxorubicin as a positive control for another 24 h. After that, the cells then fixed with 10% trichloro acetic acid for 30 min at 4 °C, followed by drying in oven 50 °C for 1 h and staining for 30 min at room temperature with 4 mg/mL SRB solution. Afterwards, the cells were washed with 1% acetic acid for 4 times, followed by drying in oven 50 °C for 1 h and dissolved with 200 µL 10 mM buffered Tris base pH 8. Cell viability was measured by the optical density at 515 nm. The wells without samples were used as negative controls.

Percent viability was calculated as follows:

$$\textit{cell viability}\,(\%) = \frac{\mathrm{OD}(\textit{cell} + \textit{sample}) - \mathrm{OD}(\mathrm{media})}{\mathrm{OD}(\mathrm{cell}) - \mathrm{OD}(\mathrm{media})} \times 100\%$$

OD = Optical Density

While the IC_{50} was calculated by linear regression analysis between percent viability and concentration [16]. The IC_{50} value of Doxorubicin as an anticancer

Table 2. ^{13}C-NMR, HMQC and HMBC data of Isobractatin

C No	^{13}C-NMR (δ ppm)	HMQC (δ^{1}H, ppm)	HMBC (δ ^{13}C, ppm)
1	166.28		
2	92.72	5.99 (1H, *s*)	166.28; 168.38; 101.43; 112.57
3	168.38		
4	112.57		
4a	156.11		
5	84.70		
6	203.88		
7	47.20	3.48 (2H, *t*, *J*=4.90; 4.85 Hz)	49.47; 203.88; 134.23
8	134.23	7.46 (1H, *d*, *J*=7.35 Hz)	203.88; 179.07; 47.20; 91.00
8a	135.28		
9	179.07		
9a	101.43		
10a	91.00		
11	43.54		
12	91.78	4.52 (1H, *q*, *J*=6.70 Hz)	112.57; 28.32; 20.18
13	13.63	1.38 (3H, *d*, *J*=6.10 Hz)	91.78; 43.,54
14	20.18	1.49 (3H, *s*)	112.57; 91.78; 43.54; 28.32
15	28.32	1.40 (5H, *d*, *J*=6.75 Hz)	112.57; 91.78; 43.54
16	26.12	2.31 (1H, *d*, *J*=4.90 Hz)	47.22; 84.70; 133.76
17	117.69	4.34 (1H, *t*, *J*=6.75 Hz)	16.87; 25.65
18	133.76		
19	25.68	1.35 (3H, *s*)	133.80; 117.69
20	16.87	1.06 (3H, *s*)	25.05; 25.68
21	26.06	2.34 (1H, *d*, *J*=4.90 Hz)	47.20; 84.70; 133.76
22	49.47	2.52 (1H, *t*, *J*=8.55 Hz)	29.19; 135.40; 203.88
23	83.04		
24	29.19	1.25 (3H, *s*)	83.04; 49.47
25	30.84	1.72 (3H, *s*)	83.04; 49.47; 29.19
26		13.08 (OH, *s*)	92.72; 101.43; 166.28

drug control was 16 nM on T47D cells.

RESULT AND DISCUSSION

Spectra UV, IR ^{1}H, ^{13}C-NMR and MS data

Compound (1): white crystal (needles) 125 mg, melting point 138-140 °C; UV spectra at $\lambda_{max.}$ 229 nm, The IR spectra bend absorption at the frequencies (ν) 3402-3226 cm^{-1}, GC-MS (m/z=412). The ^{13}C-NMR ($CDCl_3$; 125 MHz) DEPT (δ_C=ppm); 12.29 (CH_3); 12.50 (CH_3); 19.23 (CH_3); 19.65 (CH_3); 21.32 (CH_3); 21.34 (CH_3); 21.47 (CH_2); 24.61 (CH_2); 25.65 (CH); 28.40 (CH_2); 29.18 (CH_2); 31.89 (CH); 31.90 (CH_2); 32.13 (CH_2); 36.75 (C); 37.49 (CH_2); 40.75 (CH); 39.92 (CH_2); 42.45 (C); 50.34 (CH); 42.54 (CH_2); 51.49 (CH); 56.19 (C); 57.10 (CH); 72.04 (CH); 121.95 (CH); 129.49 (C); 138.57 (C); 140.99 (C). ^{1} H-NMR ($CDCl_3$; 500 MHz) δ_C=ppm 0.69 (CH_3, *s*); 0.78 (s, 3H); 0.80 (s, 3H); 0.84 (t, 3H); 1.01 (s, H); 1.03 (d, 3H, *J*=7.3Hz);5.34 (1H, *d*, *J*=4.9 Hz); 5.15 (1H, *dd, J*=8.6; 6.1 Hz); 5.01 (1H, *dd, J*=9.15; 9.50 Hz); 3.52 (1 H, m), bulk of signals CH_2 at 1.02-2.28 ppm.

Compound (**2**) and (**3**)**:** 0.754 g yellow crystal gave molecular appear at m/z=464 (LC-MS). The spectrum UV-Vis showed the absorbance maximum peak 355; 327; 259 and 247 nm. Melting point 191-192 °C. The IR spectrum are showed at (ν) 3460 cm^{-1}, 2908-2929 cm^{-1}, 1637 and 1741 cm^{-1}. The spectra ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC data were on Tables 2 and 3.

Compound (1): stigmasterol; white crystal (needles) 125 mg, melting point 138-140 °C. The UV absorption band at λ_{max} 229 nm. The IR spectra indicated the present of hydroxyl group bend absorption at the frequencies (ν) 3402-3226 cm^{-1}, molecule weight 412 and molecule formula is $C_{29}H_{48}O$. ^{13}C-NMR contain double bond at chemical shift (δ) 121.90 and 140.90 ppm was confirmed by ^{1}H-NMR at (δ) 5.16 ppm (*dd*, *J*=7.30 Hz) and 5.12 ppm (*dd*, *J*= 9.15 Hz). The present of hydroxyl on carbon number 3 confirmed by ^{13}C-NMR at (δ) 71.8 ppm with β hydroxy. ^{1}H-NMR ($CDCl_3$, 500 MHz) have four methyl singlet at (δ_H, ppm); 0.69 (3H, *s*); 0.78 (3H, *s*); 0.80 (3H, *s*) and 1.01 (3H, *s*), one methyl triplet at 0.84 and one methyl doublet at 1.03 ppm. Appearance multiplet at 3.52 ppm was indicated axial oxymethine proton. Suggesting the usual equatorial (β) orientation for hydroxyl group at C-3 this was supporting by ^{13}C-NMR signal at δ 72.04 ppm which agreed closely with that recorded for C-3

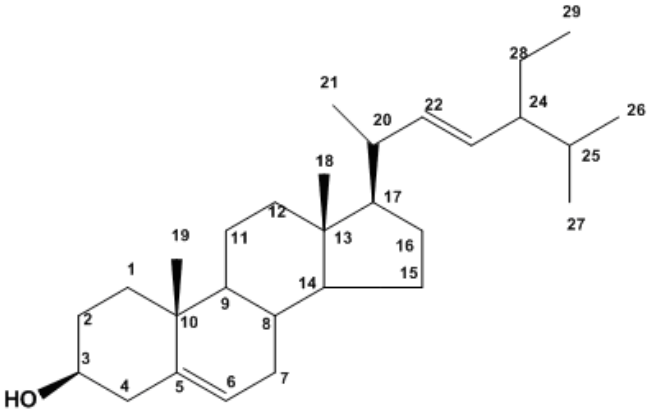

Fig 1. Stigmasterol

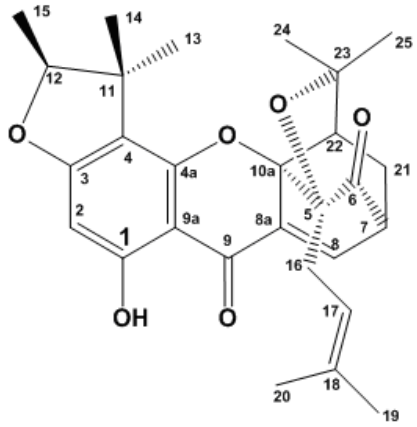

Fig 2. Isobractatin

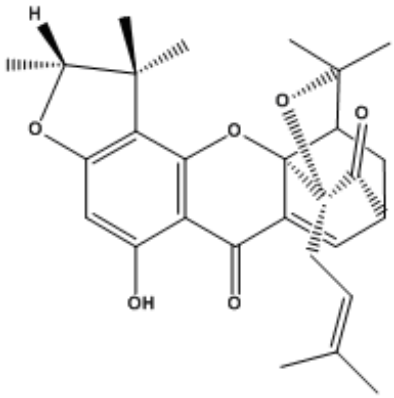

Fig 3. epi-Isobractatin

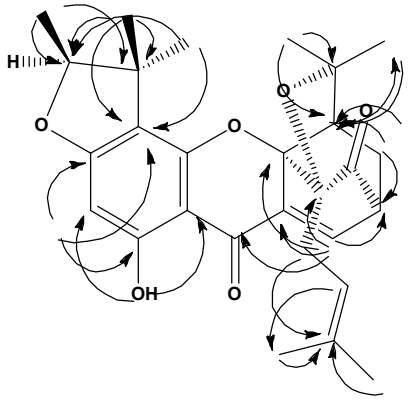

Fig 4. HMBC correlation of Isobractatin

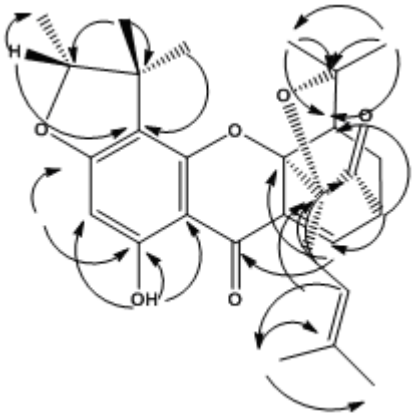

Fig 5. HMBC correlation of epi-Isobractatin

carbon with β -hydroxyl group. Base on spectroscopic data (UV-Vis, FT-IR, ^{1}H-NMR and ^{13}C-NMR) and compared with authentic sample also compared with literature data as Table 1 [17], that compound concluded as stigmasterol.

Compounds (2) and **(3**): 0.754 g yellow crystal gave molecular peak at m/z at 464 (LC-MS) corresponding to the molecule formula $C_{28}H_{33}O_6$. The spectrum UV-Vis showed the absorbance maximum peak at 355; 327; 259 and 247 nm, peaks at that absorbance showed that compound have xanthone skeleton [18]. Melting point 191-192 °C. IR spectrum exhibited absorption bands at 3460 cm^{-1} (hydroxyl group), indicated the presence of two ketone function are showed at (ν) 1637 (*ortho* hydroxyl chelated carbonyl group) and 1741 cm^{-1} (unconjugated carbonyl group). Both of these compounds can be identified because it can be tracked through proton and carbon NMR data from HMQC and HMBC were quite clear. Spectrum ^{1}H-NMR and ^{13}CNMR of compound **2** and **3** are very similar, except for the for the carbon number 12 side chain orientation. The ^{1}H-NMR each showed one methyl doublet at C12 δ 1.38 ppm (3H, *d, J*=6.10 Hz) (**2**) and 1.27 ppm (3.H, *d, J*=6.75 Hz) (**3**), showed six methyls singlet at δ 1.49 (3H, 14), 1.14 (3H, 15), 1.35 (3H, 19), 1.06 (3H, 20), 1.28 (3H, 24), 1.72 (3H, 25) (**2**); at δ 1.27 (3H, 14), 1.58 (3H, 15), 1.35 (3H, 19), 1.04 (3H, 20), 1.25 (3H, 24), 1.72 (3H, 25) (**3**). The signals methyl are showed at δ. An isophrenyl group was deduced from signals at δ 2.34 (2H, 16), 4.32 (t, H, 17), 1.35 (3H, 19) and 1.06 (3H, 20) for compound **2**;

Table 3. ^{13}C-NMR, HMQC and HMBC data of epi-Isobractatin

C No	^{13}C-NMR (δ ppm)	HMQC (δ^{1}H, ppm)	HMBC (δ ^{13}C, ppm)
1	166.45		
2	92.79	5.98 (1H, *s*)	166.45; 168.39; 101.43; 112.57
3	168.39		
4	113.70		
4a	156.63		
5	84.90		
6	203.88		
7	47.22	3.48 (2H, *t*, *J*=4.90; 4.85 Hz)	49.47; 203.88; 134.23
8	134.23	7.45 (1H, *d*, *J*=6.70 Hz)	203.88; 178.96; 47.22; 90.95
8a	135.40		
9	178.96		
9a	101.43		
10a	90.95		
11	43.30		
12	91.12	4.36 (1H, q, *J*=7.95 Hz)	24.02; 21.17
13	16.48	1.27 (3H, *d*, *J*=6.75 Hz)	91.12; 43.30
14	24.02	1.58 (3H, *s*)	91.12; 43.30
15	21.17	1.16 (3H, *s*)	113.70; 91.12; 43.30; 24.02
16	26.12	2.31 (1H, *d*, *J*=4.90 Hz)	84.90; 133.80
17	117.69	4.36 (1H, *t*, *J*=6.75 Hz)	17.01; 25.65
18	133.80		
19	25.65	1.35 (3H, *s*)	133.80; 117.69
20	17.01	1.04 (3H, *s*)	133.80; 117.69
21	26.06	2.34 (1H, *d*, *J*=4.90 Hz)	47.22; 83.11; 133.80
22	49.57	2.53 (1H, *t*, *J*=8.55 Hz)	29.06; 135.40; 203.88
23	83.11		
24	29.06	1.25 (3H, *s*)	83.11; 49.57
25	31.04	1.72 (3H, *s*)	49.57; 83.11
26		13.14 (OH, *s*)	92.79; 01.43; 166.45

Table 4. Bioassay data of cell line T47D

No	Concentration (µg/mL)	Log Concentration	% average life cell	SD	IC_{50}
1	100	2.0	3.10	0.53	
2	25	1.4	24.47	3.17	
3	10	1.0	74.38	3.02	17 µg/mL
4	5	0.70	87.77	11.0	
5	0	0	100	100	

signals at δ 2.32 (2H, 16), 4.36 (t, H, 17), 1.35 (3H, 19) and 1.03 (3H, 20) for compound **3**. Contain one OH chelated at δ 13.08 (**2**) and 13.14 ppm (**3**), one proton aromatic at δ 5.98 (H, 2), (**2**) and 5.95 ppm (H, 2) (**3**). The HMBC correlation of compound (**2**) and (**3**) showed at Fig. 4 and 5.

Cytotoxic Activity

The result showed that the IC_{50} value of the mixture of isobractatin and its epimer was 17 µg/mL. The graphic of concentration vs. cells viability (Fig. 6) showed that increasing of samples concentration significantly decreases cells viability compared to control ($P<0.05$). Based on the result, the mixture of isobractatin and its epimer has cytotoxic activity against T47D breast cancer cell. Recently, the biological activities containing anti-inflammatory, anti-bacterial, and anti-cancer effects of prenylated xanthone derivatives have been studied [19]. Four phrenylated xanthones from the pericarps of mangosteen (*G. mangostana*) strongly inhibit the cancer cells growth. The anti-proliferative effects of the xanthones were associated with cell-cycle arrest by affecting the expression of cyclins, cdc2, and p27, G1 arrest, and S arrest. The xanthones also showed apoptosis induction through the activation of intrinsic pathway following the down-regulation of signaling cascades involving MAP kinases and the serine/threonine kinase Akt [20]. Gaudichaudione A, a xanthone obtained from *G. gaudichaudii*, activated caspase-3 and induced the apoptosis of Jurkat human leukemic cells [21]. Isobractatin isolated from *G. bracteata* showed cytotoxic effect against KB cells [6].

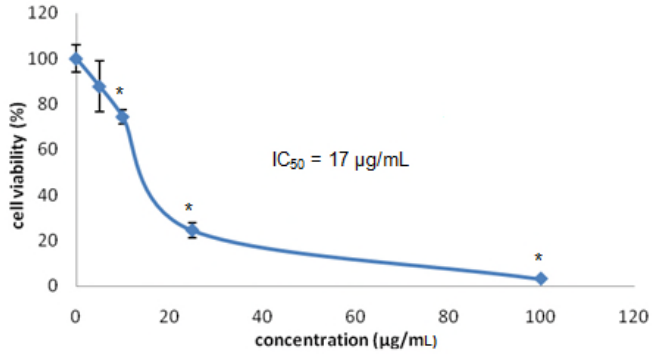

Fig 6. Percentage of viable cells of the mixture of isobractatin and its epimer in various concentrations after 24 h. Samples are conducted in triplicate and represented in mean ± standard deviation. * represented significantly differences compare to control ($P < 0.05$)

We suggest that isobractatin from *G. eugenifolia* also inhibit T47D breast cancer cells growth through this pathway. Nevertheless, further investigation is needed to explore the mechanism of cytotoxic activity of isobractatin on T47D cancer cell.

CONCLUSION

Similar to several other *Garcinia species* such as *G. morrela, G. hanburyi, G. gaudichaudii, G. bracteata*, *G. cantlyana* and *G. eugenifolia* also elaborated caged-xanthones [22]. From this investigation, the xanthone from *G. eugenifolia* seemed to be derived from mono–prenylated xanthone as mixture compound of epimer isobractatin. The mixture compound of epimer isobractatin showed cytotoxic activity against T47D breast cancer cell line with IC_{50}=17 µg/ mL.

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