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To cite this article: R Mutiah *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **456** 012030

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# New compounds of pregnanone from *Calotropis gigantea* roots actively against colon cancer cell WiDr through cell cycle inhibition

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**Abstract.** *Calotropis gigantea* (L.) W. Aiton (*C. gigantea*) is a medicinal plant that has been empirically proven to have anticancer activity. In a previous study, it showed that the fraction of ethyl acetate from the root part of *C. gigantea* had higher anticancer activity than the other fractions. It suspected that the ethyl acetate fraction of *C. gigantea* root contained active compounds that has anticancer properties. This study aimed to determine the anticancer activity of active compounds from the ethyl acetate fraction of *C. gigantea* root regarding induction of apoptosis, cell cycle arrest, and expression of caspase-8 colon cancer cell WiDr. Isolation of the active compounds from the ethyl acetate fraction of *C. gigantea* root was carried out using Bioassay-guided Isolation method. Identification of active compounds was using NMR-1H, NMR-13C, HMBC, HMQC and UPLCMS/MS methods. The anticancer activity test of the identified compounds performed by using MTT method. The induction of apoptotic and cell cycle arrest evaluated by a flow cytometry method. The result of this study showed two active compounds were identified namely (1) (Pregnanon-5-en, 3,14,17 trihydroxy-12- (4'-cyclohexyl benzoate) -, (3 $\beta$ , 12 $\beta$ , 14 $\beta$ ) - (9CI), (2) Pregn-5-en-20-one, 3,8,14-trihydroxy-12 - [(4'-hydroxy benzoyl) oxy] -, (3 $\beta$ , 12 $\beta$ , 14 $\beta$ , 17 $\alpha$ ) - (9CI). Both compounds inhibited the growth of colon cancer cell WiDr with IC<sub>50</sub> values respectively were 15.89  $\mu$ g/mL and 0.77  $\mu$ g/mL. Both compounds increased the induction of apoptotic by increasing sub-G1, S, and G2-M following depletion of G0-G1 phase accumulation.

## 1. Introduction

*Calotropis gigantea* (L.) W.T Aiton (*C. gigantea*) is a medicinal plant that grows in Indonesia. This plant has been traditionally used by Indonesian people as medicine to cure itching, scabies, boils, cough, trachoma, constipation (using its leaves), asthma, nausea, gastric pain (its flower), gonorrhoea, bites of poisonous snakes (its root), toothache, swelling, ear inflammation, intestinal worms and dysentery [1]. Scientific evidence of its anticancer activity reported including the methanol extracts and the chloroform fraction of its flower that showed antitumor activity in mice with ascites carcinoma [2]. Methanol extract (ME) and chloroform fraction of its root were able to inhibit the growth of ascites carcinoma [3].



The cytotoxic potential of cardenolide compounds has also reported against MCF-7 breast cancer cells, skin cancer cells KB, lung cancer cells NCL-H18 [4]. Cytotoxic potency of dichloromethane of leaves extract on breast cancer cells MCF-7 and MDA-MB-231, Hela cells, HT-29 colon cancer cells, ovarian cancer cells SKOV-3, and hepatic cancer cells Hep-G2 [5]. *C.gigantea* leaf extract increased the efficacy of 5-Fluorouracil and decreased the effectiveness of Doxorubicin in Widr colon cancer cell culture [6].

In a previous study, it has also reported that the compound calotropin from the root part has cytotoxic activity against leukemia K562 and gastric cancer 7901[7]; the ethanol extract of *C.gigantea* leaves was able to inhibit fibrosarcoma growth in vivo by the mechanism of increased caspase-3 expression [8]. *C.gigantea* root extract has higher anticancer activity than the leaves and flower parts [9]. The ethyl acetate fraction of the leaf and dichloromethane fraction had higher cytotoxic activity than the butanol and water fractions [10]. Active compounds that have been successfully isolated from *C.gigantea* roots, including *calotropin* (1), *frugoside* (2) [7], *afroside* (3), *15b-hydroxycalotropin* (4), *15b-hydroxycalactin* (5), *calactin* (6), *calotoxin* (7), *16a-hydroxycalactin* (8), *uscharin* (9), *coroglaucigenin* (10), *4'b,15b-dihydroxycalactin* (11), *15b-hydroxyuscharin* (12), *5b-hydroxycalac-tinic acid methyl ester* (13), *calactinic acid ethyl ester* (14), *15b-hydroxycalactinic acid ethyl ester* (15) [11].

In our previous study, we had reported that the ethyl acetate root fraction of *C. gigantea* had cytotoxic activity against WiDr cells (IC<sub>50</sub> 0.063 µg/mL) which was higher than those in dichloromethane (IC<sub>50</sub> 0.367 µg/mL), butanol (IC<sub>50</sub> 0.18 µg/mL) and water (IC<sub>50</sub> 2.24µg/mL) [10]. Our previous study also showed that ethyl acetate fraction of *C. gigantea* roots induced apoptosis through increased G2/M and increased expression of caspase-8 in colon cancer WiDr cell line [12].

Based on these studies it is suspected that there are active compounds in the ethyl acetate fraction of *C.gigantea* root that play a significant role in the anticancer activity. In this study, the isolation of active anticancer compounds from the fraction of ethyl acetate of *C. gigantea* root was carried out using Bioassay-guided isolation approach to WiDr cell and the working mechanism of the active compound is described through induction of apoptosis and Cell cycle arrest.

## 2. Material and methods

### 2.1. Material

Roots of *C. gigantea* obtained from the city of Malang, East Java tool-type and determination was carried out in LIPI Purwodadi, East Java, Indonesia. A specimen voucher (No. 201303) deposited at the Phytochemistry laboratory of Maulana Malik Ibrahim State Islamic University.

### 2.2. Ethical Approval

The study approved by The Ethical Committee of Faculty of Medicine, Brawijaya University, certificate number: 425-KEP-UB, Date: May 24<sup>th</sup>, 2015.

### 2.3. Extraction and Isolation of active compounds

The active compound was obtained from several stages of separation using bioassay-guided isolation approach. Dried powder of the root (5 kg) was extracted by maceration using ethanol 70%. The ethanol extract (214.5 g) was further fractionated by the liquid-liquid partition method using dichloromethane, ethyl acetate, butanol and water as solvents. The obtained fractions (F1-F4) tested for the anticancer activity in vitro using colon cancer cell WiDr. Furthermore, the most active fraction (F2) i.e. ethyl acetate fraction (16.02 g) was separated for its active compound by vacuum column chromatography using silica gel G 60 as a stationary phase and gradient mobile phase CHCl<sub>3</sub>: CH<sub>3</sub>OH (100: 100 - 50:50), where 7 subfractions (SF1-SF7) obtained. The IC<sub>50</sub> data in table 1 showed that SF3 has the lowest IC<sub>50</sub> value (6.09µg/mL). An open column chromatography further separated the most active subfraction (SF3: 280 mg) with silica gel 60 and mobile phase were hexane and ethyl acetate (4: 1); ethyl acetate: chloroform (9: 1); and chloroform: methanol 1: 1. In this separation, two single stains called compound 1 (10 mg), and compound 2 (15 mg) obtained. The results of anticancer activity assays of SF1-SF7 were presented in table 1.

#### 2.4. Identification of isolates

Mass spectra of active isolates were obtained using UP-LCMS / MS tool-type ACQUITY UPLC I-Class (Waters, United Kingdom) with detector diode-array detector (DAD) 2996 (waters); column: Sunfire C18, p 50 mm, diameter 2 mm, particle size  $\mu\text{m}$  (Waters). Mobile phase: acetonitrile gradient; water; acid format, flow rate 1ml / min, injection volume 10  $\mu\text{l}$ . MS system Xevo G2-S QTOF (Waters), analyzer: TOF with positive electrospray (ES +), gas flow 794 L/min. NMR spectra; HMQC, HMBC (for 2D NMR) obtained by NMR JEOL ECS400 (400 MHz),  $\text{CDCl}_3$  as solvent and TMS as an internal standard.

**Table 1.** Values of  $\text{IC}_{50}$  of SF1-SF7 Treatments on the Growth of WiDr Colon Cancer Cells.

Groups/dose	Mean of Viability WiDr Cells Line at treatment concentration ( $\mu\text{g}/\text{mL}$ )								$\text{IC}_{50}$ ( $\mu\text{g}/\text{mL}$ ) $\pm$ SD*
	200	100	50	25	12.5	6.25	3.75	0.935	
SF1	25.16	31.78	40.43	60.54	65.06	74.52	78.69	83.89	32.38 $\pm$ 2.32
SF2	45.43	48.78	49.54	59.26	46.69	64.42	77.59	87.92	59.53 $\pm$ 9.51
SF3	1.82	3.10	4.52	8.72	50.89	60.32	64.82	76.79	6.09 $\pm$ 0.08
SF4	3.58	7.88	15.21	31.69	55.67	69.91	73.11	86.97	11.05 $\pm$ 1.96
SF5	34.17	44.22	54.74	64.22	61.40	73.27	83.84	85.91	60.72 $\pm$ 2.74
SF6	39.41	45.78	59.88	69.69	82.07	85.27	89.11	88.91	103.27 $\pm$ 9.63
SF7	22.83	28.35	32.55	42.46	51.82	65.46	73.89	89.46	18.66 $\pm$ 2.85

\*Average value of  $\text{IC}_{50} \pm \text{SD}$  in triplicates

#### 2.5. Anticancer activity Test

Compound 1 and 2, were tested for their anticancer activity in vitro using colon cancer cells of WiDr. Anticancer activity expressed as  $\text{IC}_{50}$ . Cell viability was determined using MTT assay. The cytotoxic test stages were carried out according to the protocol of CCRC-UGM [13].

#### 2.6. The apoptotic induction test

The mechanism of action of the anticancer activity determined by three parameters: cell cycle regulation, induction of cell apoptosis and caspase-8 expression. Cell cycle regulation was analyzed using fluorescence-activated cell sorting method using propidium iodide (PI) probe. The induction of cell apoptosis also determined by using a flow cytometer with a combination of PI-Annexin V reagents. Immunocytochemical methods observed Caspase-8 expression. The stages of apoptotic induction testing performed according to the protocol of CCRC-UGM [14].

#### 2.7. Cytotoxicity Test Using MTT Assay

The suspension of colon cancer cells WiDr (100  $\mu\text{L}$ ) contains  $3 \times 10^4$  cells / 100  $\mu\text{L}$  of medium was distributed into wells on a 96-well plate and incubated for 24 hours. After incubation, 100  $\mu\text{L}$  of the test solution added into the well, at a series of concentrations. As a positive control, 100  $\mu\text{L}$  of culture medium added, then 100  $\mu\text{L}$  of cisplatin at series concentration into the wells. As a cell control, 100  $\mu\text{L}$  of culture medium attached to the well containing 100  $\mu\text{L}$  of cell suspension. For a solvent control, 100  $\mu\text{L}$  of DMSO added to the well containing 100  $\mu\text{L}$  of culture medium and 100  $\mu\text{L}$  of the cell suspension with the same serial dilution corresponding to those of the test solution. They incubated for 24 hours at 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . At the end of incubation, medium removed, and 10  $\mu\text{L}$  of MTT solution (5 mg/mL PBS) added, and the medium replaced with 190  $\mu\text{L}$  complete RPMI 1640 medium. Cells incubated for 3-4 hours. The MTT reaction stopped with the addition of SDS stopper reagent (100  $\mu\text{L}$ ). The microplate was then wrapped in tissue paper and incubated overnight in dark room at room temperature. Live cells react with MTT to form a purple color. The test results were read using ELISA reader at a wavelength of 595 nm [14].

### 2.8. Analysis of cell cycle with flow cytometry

In this cell cycle analysis, Propidium Iodide (PI) used as a dye to analyze the number of DNA sets in each cell. Cells of  $5 \times 10^5$  cell/well were incubated in 6-well plate. Cells treated with DMSO (0.25%) and active isolates at its  $IC_{50}$ . The cells were then reincubated for 24 hours. At the end of the incubation, the medium was removed and transferred into a tube and centrifuged (2000 rpm, 3 min). The supernatant removed. PBS added to the wells where the medium removed, and moved to the same microtube, centrifuged and the supernatant discarded. This step repeated once again, and the cells were harvested with trypsin. Cells were transferred into the same microtube then centrifuged at 2000 rpm, for 3 min. The rest of the harvesting cells in the well were rinsed with PBS and centrifuged again, and the PBS discarded. The cell precipitates in the microtube then fixed with 70% ethanol,  $-20^\circ\text{C}$ , incubated for 30 min at room temperature or overnight at  $4^\circ\text{C}$ , then centrifuged (2000 rpm, 3 min). The cell precipitate washed with PBS 2 times and then PI reagents were added and immediately homogenized. The microtube containing the cell suspension is wrapped in aluminum foil and incubated in a  $37^\circ\text{C}$  water bath for 20 minutes. The cell suspension was homogenized again and transferred into a flow cytometer tube using a nylon filter, and ready to be analyzed.

### 2.9. Analysis of cell apoptosis induction using flow cytometry

Cells were plated in 6-well plate ( $5 \times 10^5$  cells / well) and treated with DMSO (0.25%) and the active isolates. The cells were then re-incubated for 24 hours. At the end of the incubation, the medium was removed and transferred into a tube and centrifuged (2000 rpm, 3 min.) and then the supernatant discarded. PBS was added to the well and assigned to the same microtube, centrifuged again. The supernatant discarded. This step repeated once again, and the cells were harvested with trypsin. Cells were transferred into the same microtube and centrifuged (2000 rpm, 3 min.). The rest of the harvesting cells were rinsed with PBS and centrifuged again, and the PBS discarded. To the precipitates, PI-Annexin V reagents were added and immediately homogenized. The microtube containing the cell suspension was wrapped in aluminum foil and incubated in a water bath at  $37^\circ\text{C}$  for 5 minutes. The cell suspension was homogenized again and transferred into a flow cytometer tube using a nylon filter, and ready to be analyzed.

## 3. Results and discussion

### 3.1. Identification of Compound 1

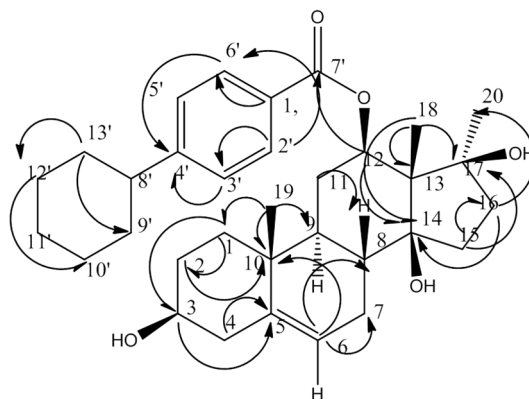
Compound 1 was a colorless gel-shaped compound. Chromatogram profile UPCLMS/MS of compound 1 with a C18 Sunfire stationary phase and a mobile phase of gradient acetonitrile: water: Formic acid gave a single peak at retention time of 14.24 minutes with the dominant area under the curve. The mass spectrum of compound 1 showing the molecular ion  $m/z$  522.3380 (measured mass), while the calculated mass of compound 1 based on ChemDraw buffer was  $m/z$  522.3345. In conclusion, the molecular formula of compound 1 is identical to  $\text{C}_{33}\text{H}_{46}\text{O}_5$ . The result from nuclear magnetic resonance spectroscopy NMR-1H showed the number of protons and chemical shift positions ( $\delta$ ) indicating the type of proton in compound 1 is  $\delta = 1.59$  (proton  $\text{CH}_3$ , 3H, singlet);  $\delta = 2.203$  ( $\text{CH}_3$ , 3H, singlet);  $\delta = 2.77$  ( $-\text{CH}$  cyclohexane, 1H, multiplet);  $\delta = 3.651$  (OH alcohol, 3H, singlet overlapped);  $\delta = 4.216$  ( $-\text{CH}$  cyclohexane, 1H, triplet)  $\delta = 5.341$  ( $-\text{CH}$  cyclohexane, 1H, singlet);  $\delta = 7.53/\text{C}3'$  ( $=\text{CH}$  benzene, 1H,  $\delta = 7.54/\text{C}5'$  ( $=\text{CH}$  benzene, 1H, triplet) triplet);  $\delta = 7.716/\text{C}2'$  ( $=\text{CH}$  benzene, 1H, triplet);  $\delta = 7.714/\text{C}5'$  ( $=\text{CH}$  benzene, 1H, triplet). The presence of cyclohexane substitution is indicated by  $\delta = 2.287/\text{C}22$  ( $-\text{CH}$ , 1H, triplet);  $\delta = 1.668/\text{C}23$  ( $-\text{CH}_2$ , quartet),  $\delta = 1.653/\text{C}27$  ( $-\text{CH}_2$ , quartet);  $\delta = 1.289$  ( $-\text{CH}_2$ , multiplet);  $\delta = 1.285$  ( $-\text{CH}_2$ , multiplet);  $\delta = 1.29$  ( $-\text{CH}_2$ , multiplet). Based on NMR-13C magnet resonance spectroscopy data and chemical shift position ( $\delta$ ) showed the type of carbon in compound 1 is :  $\delta = 167.886$  ( $-\text{C}=\text{O}$ , C carboxyl);  $\delta = 132.503$  ( $=\text{C}-\text{C}$ ; C benzene);  $\delta = 130.997$  ( $=\text{C}-\text{C}$ ; CH benzene);  $\delta = 128.890$  ( $=\text{C}-\text{C}$ ; CH benzene);  $\delta = 68.231$  ( $\text{O}-\text{C}-\text{C}$ ; CH cyclohexane);  $\delta = 41.074$  ( $\text{C}-\text{C}-\text{C}$ ; C cyclohexane);  $\delta = 38.778$  ( $\text{C}-\text{C}-\text{C}$ ; C cyclohexane);  $\delta = 34.202$  ( $\text{C}-\text{C}-\text{C}$ ; C cyclohexane)  $\delta = 32.028$  ( $\text{C}-\text{C}-\text{C}$ ; C cyclohexane)  $\delta = 30.414$ ;  $\delta = 29.797$ ;  $\delta = 29.463$ ;  $\delta = 29.358$ ;  $\delta = 29.253$ ;

$\delta=29.215$ ;  $\delta=29.006$ ;  $\delta=27.229$ ;  $\delta=25.039$  (CH<sub>2</sub> cyclohexane);  $\delta=23.800$ ;  $\delta=23.085$ ;  $\delta=22.798$  (CH<sub>2</sub> cyclopentane);  $14.179$  (CH<sub>3</sub> aliphatic);  $11.061$  (CH<sub>3</sub> aliphatic). The proton and carbon shifts showed that compound 1 is identical to *pregnanon-5-en, 3,14,17 trihydroxy-12-(4'-cyclohexylbenzoyl)oxy-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ )-(9CI)*.

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**Table 2.** The chemical shift of carbon and proton of isolates of compound 1 and the correlation of carbon with protons.

C	$\delta$ H (400 Hz)	$\delta$ C (400 HZ)	HMBC
1	1.745 (1H, m), 1.11 (1H,m)	30.41	C2, 3, 5, 10
2	1.594, 1.30 (1H,m)	29.46	C3, 4, 10
3	3.65 (2H, s), 3.45 (1H,s)	68.231	C1, 2, 5
4	2.306 (1H, dd), 2.25 (1H, overlapped)		C2, 6, 5, 10
5	.		
6	5.361 (1H, m)		C4, 5, 7, 10
7	2.26, 1.984 (each 1H, m, J=0.47)	25.8	C5,6,9, 14
8	1,844 (1H,m)	34.2	C7, 10, 14
9	1.316 (1H, m)	38.77	C1, 5, 11, 12,14
10		29.79	
11	2.001, 1.984 (1H,m, overlapped)	23.08	C8, 10,13
12	4.229 (1H, dd)		C7'
13		41.07	
14		51.57	
15	2.15, 1.98 (each 1H,m)	29.21	C8, 13,16,17
16	2.806 (1H,m), 1.88 (1H,m)	29.25	C13,14,20
17		68.23	
18	1.30 (3H,S)	11.06	C-12,13,14
19	0.903 (9 H, m)	14.18	C-1,5,9,10
20	1.29 (1H,s)	27.29	
8'	2.287 (triplet)	41.08	
9', 13'	1.668; 1.653 (2H,dd)	29.0	C-12',C9', C10'
10', 12'	1.289; 1,285 (13H, dd)	22.7	C-9', C10
11'	1.29 (13H,m)	23.8	
1'		132.503	C2', 6'
2', 6'	7.716 (1H,d)	130.99	C1',3', 4',6'/2'
3', 5'	7.52(1H,t)	128.89	C1',2', 4', 5'/3'
4'		128.117	C1', 2', 5',6'
7'		167.886	



**Figure 1.** HMBC chemical structure of compound 1: pregnanon-5-en, 3,14,17 trihydroxy-12-(4'-cyclohexylbenzoyl)oxy-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ )-(9CI).

### 3.2. Identification of Compound 2

Compound 2 was a white needle crystal. The mass spectrum of compound 2 showed the measured mass at  $m/z$  484.2460. The calculated mass of compound 2 was  $m/z$  484.2461 which corresponds to the molecular formula of  $C_{28}H_{36}O_7$ . The nuclear magnetic resonance spectroscopy NMR-1H showed the number of protons and the chemical shift position ( $\delta$ ) indicated the proton type in compound 2 is  $\delta/C2',6' = 7.71$  (=CH benzene, 1H, dd),  $\delta/3',5' = 7.52$  (=CH benzene, 1H, dd), the presence of OH in 4' benzene carbon is shown by  $\delta/C4' = 5.93$  (=OH benzene, 1H, s);  $\delta/C1 = 1.61; 1.23$  (-CH<sub>2</sub> cyclohexane, m),  $\delta/C2 = 1.61; 1.26$  (-CH<sub>2</sub> cyclohexane, m). The presence of a hydroxyl proton at the pregnanone core indicated by the shift of the proton at  $\delta/C3 = 3.47$  (-OH cyclohexane, 1H, singlet);  $\delta/C8, C14 = 3.65$  (-OH cyclohexane, 2H, singlet), the presence of a methyl proton at the pregnanone core was indicated by  $\delta/C6 = 5.27$  (=CH cyclohexane, 1H, s),  $\delta/C4, C7, C15, C16 = 2.035$  (-CH<sub>2</sub> cyclohexane, 4 H, m),  $\delta = 4.22$  (-CH-COO, triplet),  $\delta = 2.612$  (-CH-CO cyclopentane, triplet). The chemical shift and the number of protons were identical to the compound *pregn-5-en-20-one, 3,8,14-trihydroxy-12-[(4'-hydroxy benzoyl)oxy]-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-(9CI)*. Based on the number of protons, the position of the proton chemical shift, and molecular weight and the fragmentation of molecular ions, it can be concluded that compound 2 (SF 2.4.2) was identical with the compound of *pregn-5-en-20-one, 3,8,14-trihydroxy-12-[(4'-hydroxy benzoyl)oxy]-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-(9CI)*. The chemical shift and structure of the compound are presented in Table 3 and Figure 2. The chemical shift and structure of the compound have similarities to the compounds in the previous studies [15].

### 3.3. Anticancer activities of Compound 1 and 2

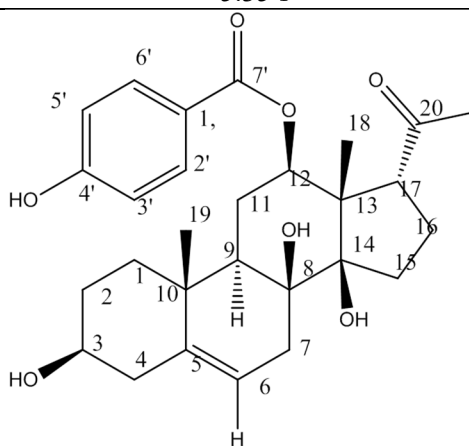
The effectiveness of anticancer of compound 1 and 2 tested by MTT assay. Table 1 shows the increased concentration of the compound 1, ie. (*pregnanone-5-en, 3,14,17 trihydroxy-12-(4'-cyclohexyl benzoyl)oxy-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ )-(9CI)*) and compound 2 ie. *pregnanone-5-en-20-one, 3,8,14-trihydroxy-12-[(4'-hydroxy benzoyl)oxy]-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-(9CI)* can lead to decreased viability of WiDr cells. From the value of IC<sub>50</sub>, it showed that compound 2 has a stronger anticancer activity than compound 1. The percent viability of WiDr cells and IC<sub>50</sub> average of compound 1 and compound 2 presented in table 4.

### 3.4. Cell cycle inhibition

To determine whether the antiproliferative effect caused by the treatment of compounds 1 and two that leads to a resting phase, further analysis of changes in the distribution of WiDr cancer cell cycle is required. Cell cycle analysis was performed by fluorescence-activated cell sorting method using propidium iodide (PI) probe. The results of cell cycle analysis of WiDr cancer due to the treatment of compound 1 and compound 2 are presented in Figure 3.

**Table 3.** Proton shift of compound 2 of ethyl acetate fraction of the *C.gigantea* root.

H	Compound 2	Reference [15]
1	1.61 m; 1.23 m	1.72 m; 121 m
2	1.61 m; 1.26 m	1,75 m; 125 m
3	3.65 m	3.69 m
4	2.31 m ; 2.03	2.31 m; 2.03 m
6	5.27 s	5.28 brs
7	2.03 m	2.03 m
8	3.65 s	3.65 s
9	1.32 dd	1.46 dd
11	1.88 m; 1.61 m	1.88 m; 1.67 m
12	4.22 dd	4.74 dd
14	3.65 s	3.65 s
15	2.03 m; 1.58 m	1.99 m; 1.58 m
16	2.03 m; 1.61 m	2.03 m; 1.63 m
17	3.4 s	3.2 s
18	1.26 s	1.59 s
19	0.92 s	1.03 s
21	2.03 s	1.96 s
2',6'	7.71 dd	7.86 dd (8.0, 1.6)
3',5'	7.52 dd	7.52 dd
4'	5.9 s	5.35 s

**Figure 2.** Chemical Structure of Compound 2: Pregnanone-5-en-20-one, 3,8,14-trihydroxy-12-[(4'-hydroxybenzoyl)oxy]-, (3 $\beta$ ,12 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-(9CI).**Table 4.** Percent of WiDr cell viability and IC<sub>50</sub> of compound 1 and compound 2.

Compound	Cell viability (%) $\pm$ SD at treatment concentration ( $\mu$ g/ml)					IC <sub>50</sub> ( $\mu$ g/ml) $\pm$ SD
	0	0.78	12.5	25	50	
1	100 $\pm$ 0	27.37 $\pm$ 1.04	33.54 $\pm$ 1.04	42.31 $\pm$ 2.45	49.64 $\pm$ 9.27	15.89 $\pm$ 1.36
2	100 $\pm$ 1	70.01 $\pm$ 0.16	23.28 $\pm$ 3.01	26.41 $\pm$ 8.53	26.68 $\pm$ 4.16	3.18 $\pm$ 0.69



### 3.5. Cell cycle inhibition

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### 3.6. Induction of apoptosis

Observation of apoptotic induction was performed to determine the cause of cell death for both apoptosis and necrosis. The flow cytometry method can distinguish living cells, early apoptosis, late apoptosis, and necrosis because the reagents Annexin V and PI work selectively bind to whole cells or those that not intact (fragmentation). The results of the apoptotic induction test with flow cytometry presented in Figure 4. Inhibition of proliferation may be caused by modulation in cell cycle and by the induction apoptosis. The data of IC<sub>50</sub>, G2 / M phase, apoptosis of colon cancer cell WiDr as the results of treatment with compound 1 and 2 presented in table 4.

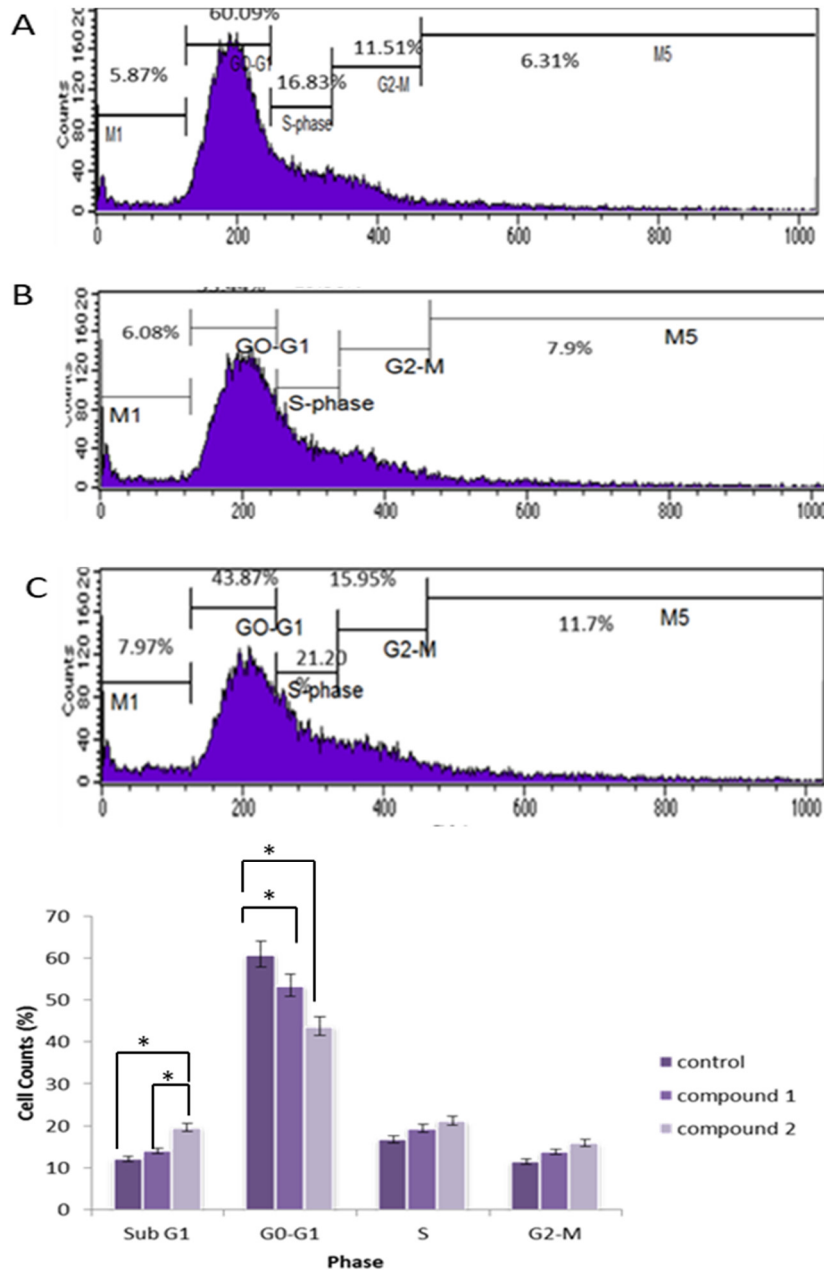
The purpose of isolation and identification of anticancer compounds in this study was to obtain an active anticancer compound from *C. gigantea* root F2. This isolation identified by using bioassay-guided isolation method, i.e., separation of an active anticancer compound from *C.gigantea* root extract which guided its anticancer activity from fraction, subfraction up to its isolate. Isolates were known to be active as anticancer identified by their chemical structure using nuclear magnetic resonance spectra of proton and carbon (NMR-1H, NMR-13C) recorded with JOEL ECS-400 spectrometer and electrospray mode (ES +) mass spectra obtained by UPLCMS/MS.

Anticancer activity test was performed in vitro using colon cancer cell WiDr. Selection of cancer cells based on previous research where there was still no report on the anticancer activity of an active compound from the root *C.gigantea*. The characteristic of these cancer cells was the high expression of cyclooxygenase-2 (COX-2) that stimulates the proliferation of WiDr cells [16]. In the WiDr cell, there was a p53 G mutation at a position of 273 resulting in a change in arginine residue to histidine [17]. However, p21 in normal WiDr cells allows for the cessation of cell cycles [18]. Apoptosis in WiDr cells can occur via the independent pathway of p53, including through p73 activation [19]. Based on the results of the above analysis, this study found 2 active anticancer compounds consisting of one novel compound (pregnanone-5-en, 3,14,17 trihydroxy-12- (4'-cyclohexyl benzoyl) -, (3 $\beta$ , 12 $\beta$ , 14 $\beta$ ) - (9CI), and 1 known compound (1) Pregn-5-en-20-one, 3,8,14-trihydroxy-12 - [(4'-hydroxy benzoyl) oxy] -, (3 $\beta$ , 12 $\beta$ , 14 $\beta$ , 17 $\alpha$ ) - (9CI) . The two compounds belonged to the triterpenoid group with the pregnanon core and may inhibit the growth of colon cancer cell WiDr by induction of apoptosis and cell cycle inhibition.

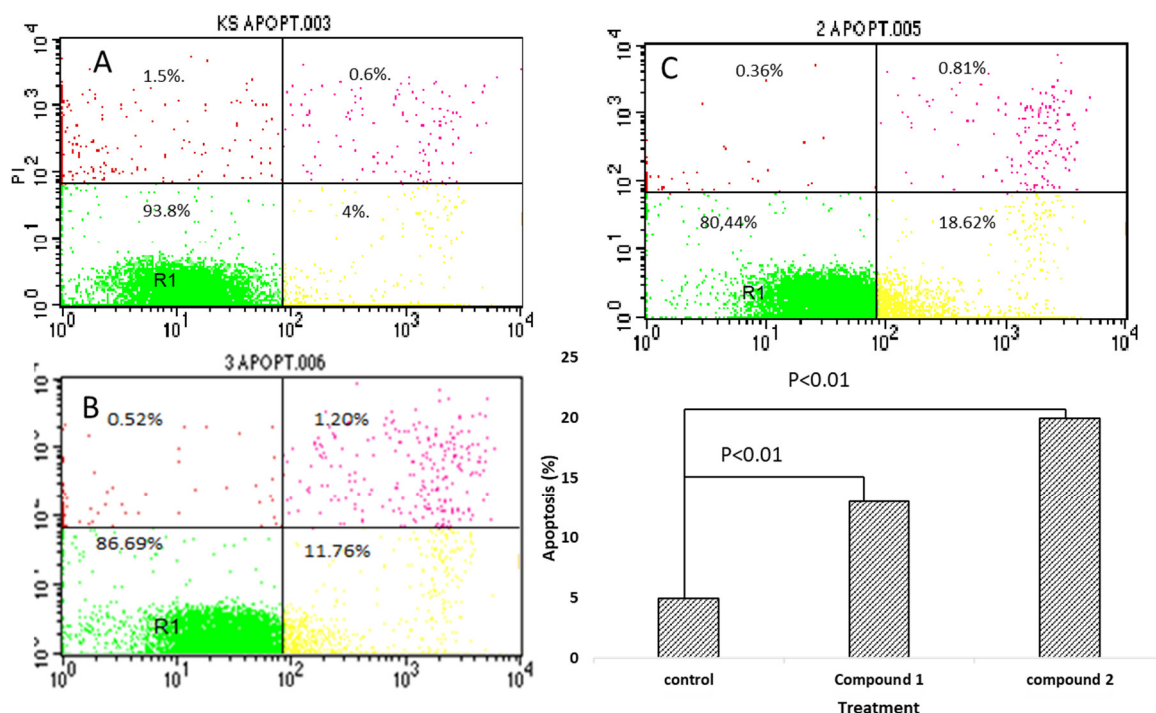
Treatment at the dosage of IC<sub>50</sub> for compound 1 (15.89  $\mu$ g / ml) and compound 2 (3.81  $\mu$ g / ml) in the proliferation of WiDr cells caused cell cycle changes with increasing of sub-G1, S, and G2-M following depletion of G0-G1 phase accumulation compared to control cells (Fig 3). Distribution changes in the cell cycle after the treatment will direct the cell to rest at a particular phase in the cell cycle, causes the induction of cell death. Those against cancer cell WiDr created increase apoptosis consecutively 19.43% and 12.96% compared to control (4.6%). The increase of apoptotic induction of both compounds was significantly different from control (p <0.001).

The decreased of the cell accumulation in G0-G1 phase colon cancer cell WiDr due to the therapy of compound 1 and compound 2, was suspected result of the apoptosis induction by both compounds through activation of P53. In previous studies, it has found that triterpenoid compounds can induce apoptosis through activation of P53 [19]. At the G1/S checkpoint, the damage of DNA could trigger cell cycle arrest, and this process was p53-dependent. In general, the low levels of p53 in a cell were negatively regulated by MDM2 which targeting the degradation of p53, but DNA damage could induce p53 activity rapidly [20]. P53 is a transcriptional factor in the formation of p21. Increased p21 will suppress all CDC (Cyclin-Dependent Kinase) with cyclin, where the cell division cycle is highly dependent on complex bonds between CDK and cyclin. In case of binding of p21, all CDK will be

suppressed both CDK-1 in phase M and CDK-4 and CDK-6 in phase S, the cell cycle will stop so that p53 will trigger Bax activity. Bax protein will suppress the activity of Bcl-2; therefore the mitochondria membrane permeability changes resulting in a release of cytochrome c into the cytosol and it will activate caspase cascade. This active caspase will activate the DNase, penetrate the nuclear membrane, and damage the DNA so that the DNA will be fragmented and apoptotic [21].



**Figure 3.** The induction effect of cell death and changes in distribution at the cell cycle phase against WiDr cells as results of treatment at the dose of IC<sub>50</sub> with compound 1 and compound 2. Statistically significant differences (p<0.01) as compared to control in Sub G1 and G0-G1 phases. (A) control; (B) compound 1; (C) compound 2.



**Figure 4.** Cell distribution and analysis of cell death induction using Flowcytometry (A) control of WiDr cell (B) treatment with compound 1; (C) treatment with compound 2 against WiDr cells: (R1) live cell (R2) cell apoptosis (R3) late apoptosis, and (R4) necrosis.

**Table 4.** IC<sub>50</sub> data, G2/M phase, apoptosis of colon cancer cell WiDr due to treatment with compound 1 and 2.

Compound	IC <sub>50</sub> (µg/ml)	Sub G1	G2-M Phase (%)	S Phase	Apoptosis Cell (%)
Control		12.8	9.29	16.83	4.6
<i>Pregnanon-5-en, 3,14,17 trihydroxy-12-(4'-cyclohexylbenzoyl)oxy-, (3β,12β,14β)-(9CI)</i>	15.89	13.98	12.48	19.38	19.43
<i>Pregn-5-en-20-one, 3,8,14-trihydroxy-12-[(4'-hydroxybenzoyl)oxy]-, (3β,12β,14β,17α)-(9CI)</i>	3.18	19.67	15.55	21.2	12.86

#### 4. Conclusion

Two active compounds from the ethyl acetate fraction of *C.gigantea* root were obtained consisting of one novel compound *pregnanone-5-en, 3,14,17 trihydroxy-12-(4'-cyclohexyl benzoyl)oxy-, (3β,12β,14β)-(9CI)*, and one known compound that has not been reported found in *C.gigantea*, *pregnanone -5-en-20-one,3,8,14-trihydroxy-12- [(4'-hydroxy benzoyl)oxy]-, (3β,12β,14β,17α)-(9CI)*. Compound 1 (IC<sub>50</sub>: 15.89 µg/ml) and 2 (IC<sub>50</sub>: 3.18 µg/ml) may induce apoptosis of colon cancer cell WiDr with increasing of sub-G1, S, and G2-M following depletion of G0-G1 phase accumulation

compared to control cells. Both compounds may potentially be developed as anticancer agent candidates.

### Acknowledgments:

We sincerely thank the Directorate General of Islamic Higher Education (DIKTIS) for their financial support through “Hibah Kompetitif” Research Grant in 2016 that made this study possible.

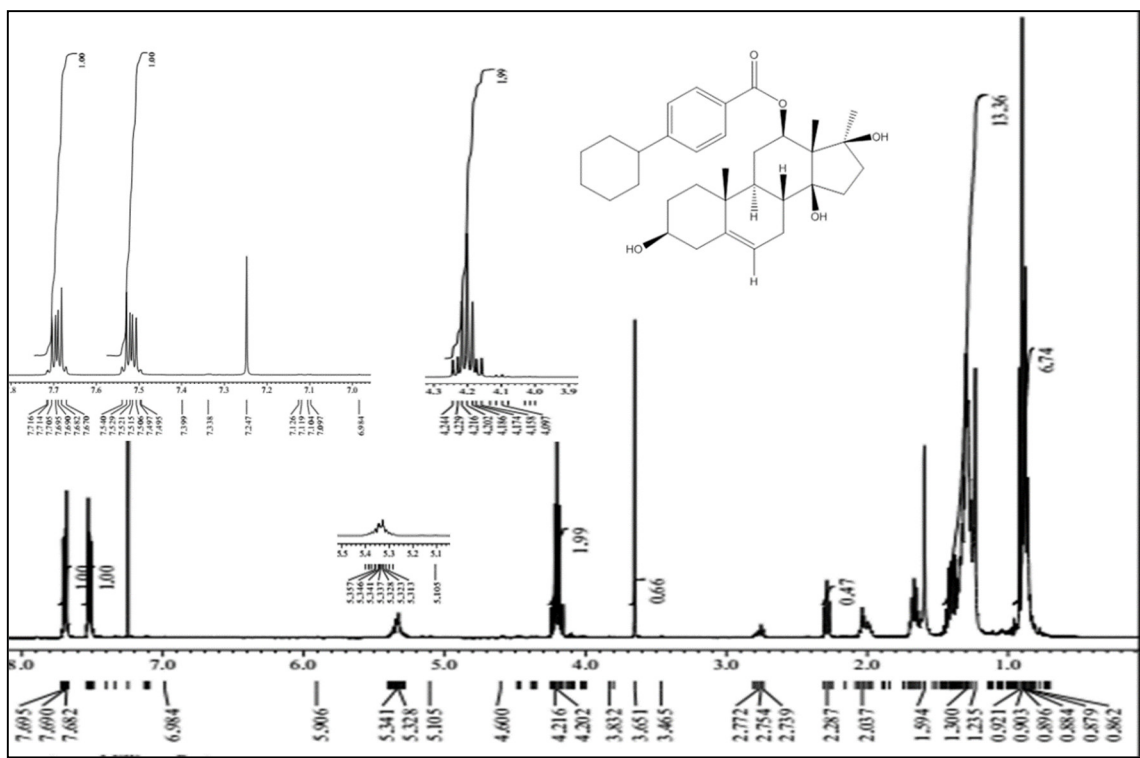
### Supplementary material

Includes the section on experimental procedures and Figures S1–S9

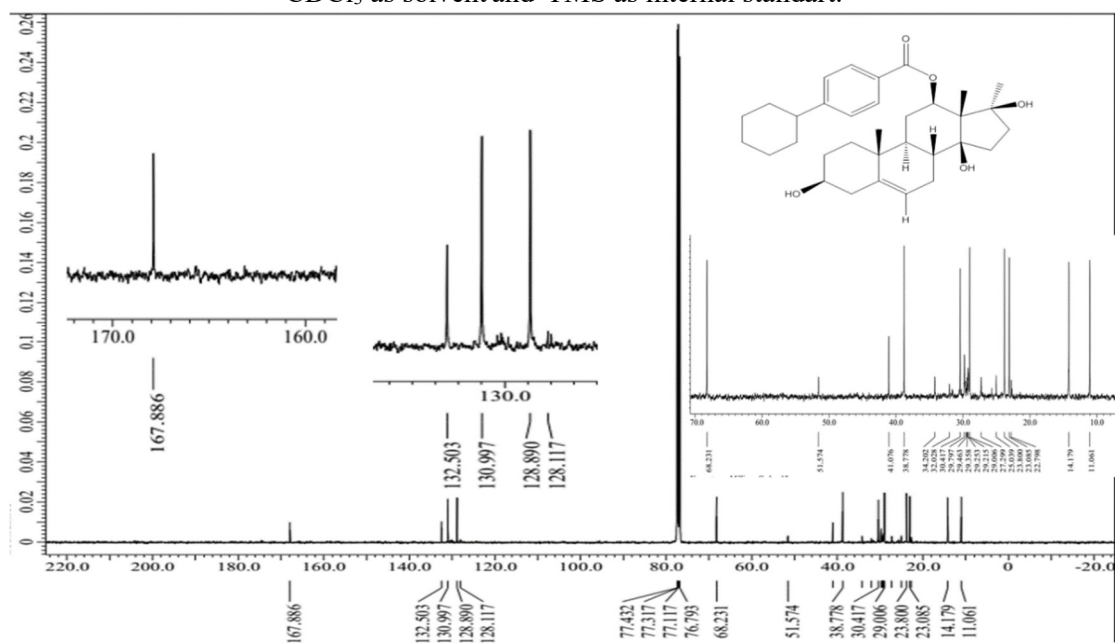
### References

- [1] Radjakmanugunsudarso M 1968 *Cabe Puyang Warisan Nenek Moyang* 2th ed (Jakarta: Balai Pustaka)
- [2] Habib M R, Aziz M A and Karim M R *J Appl Biomed* **8** 47-54
- [3] Habib M R and Karim M R 2011 *Asian Pac J Trop Med* **4** 786-90
- [4] Seeka C and Sutthivaiyakit S 2010 *Chem Pharm Bull* **58** 725-28
- [5] Wong S K, Lim Y Y, Abdullah N R and Nordin F J 2011 *BMC Complement Altern Med* **11** 3
- [6] Mutiah R, Widyawaruyanti A and Sukardiman S 2018 *JAPS* **8** 51-56
- [7] Wang Z N, Wang M Y, Mei W L, Han Z and Dai H F 2008 **13** 3033-39
- [8] Mutiah R, Griana T P, Ula Q N and Andhyarto Y 2016 *Int J Pharm Clin Res* **8** 167-71
- [9] Mutiah R, Widyawaruyanti A, Zulaikah S and Sukardiman 2016 *Alchemy* **18** 1-4
- [10] Mutiah R, Sukardiman and Widyawaruyanti A 2017 *Int J Pharm Pharm Sci* **9** 83-86
- [11] You H, Lei M, Song W, Chen H, Meng Y, Guo D, Liu X and Hu L 2013 *Steroids* **78** p 1029-34
- [12] Mutiah R, Widyawaruyanti A and Sukardiman S 2017 *JAPS* **7** 197-201
- [13] Mutiah R 2014 *Pengembangan Fitofarmaka Antikanker (Panduan Teknik Pengembangan Obat Herbal Indonesia Menjadi Fitofarmaka)* 1th ed (Malang: UIN Maliki Press)
- [14] Cancer Chemoprevention Research Center Farmasi UGM (CCRC) 2009 *Prosedur Tetap Pengamatan Ekspresi Protein Dengan Metode Imunositokimia* available at <http://ccrc.farmasi.ugm.ac.id/wp-content/uploads/12-imunositokimia1/>>
- [15] Ibrahim S M R, Mohamed G A, Shaala L A, Banuls L M Y, Kiss R and Youssef D T 2015 *Steroids* **96** 63-72
- [16] Palozza P, Serini S, Maggiano N, Tringali G, Navarra P and Ranelletti F O 2005 *J. Nutr.* **135** 129-36
- [17] Noguchi P, Wallace R, Johnson J, Earley E M, O'Brien S, Ferrone S, Pellegrino M A, Milstien J, Needy C and Browne W P J 1979 *In Vitro* **15** 401-08
- [18] Han C L, Chen G G, Vlantis A C, Leung B C S, Tong M C F and Van Hasselt C A 2006 *Cancer J.* **12** 482-93
- [19] Liu Y, Whelan R J, Pattnaik B R, Ludwig K, Subudhi E, Rowland H, Claussen N, Zucker N, Uppal S, Kushner D M, Felder M, Patankar M S and Kapur S A 2012 *Plos. One* **7** 1-10
- [20] Lapenna S and Giordano A 2009 *Nat. Rev. Drug Discov.* **8** 547-66
- [21] Reed S I 2011 Cell Cycle. In: DeVita, Hellman, Rosenberg's. *Cancer Review- Principles and Practice of Oncology review* 9th ed pp 75-83

## SUPPLEMENTARY MATERIAL



**Figure S1.**  $^1\text{H}$ -NMR spectra of compound 1 analyzed with NMR JEOL ECS400 (400 MHz) using  $\text{CDCl}_3$  as solvent and TMS as internal standart.



**Figure S2.**  $^{13}\text{C}$ -NMR compound 1 analyzed with NMR JEOL ECS400 (400 MHz) using  $\text{CDCl}_3$  as solvent.

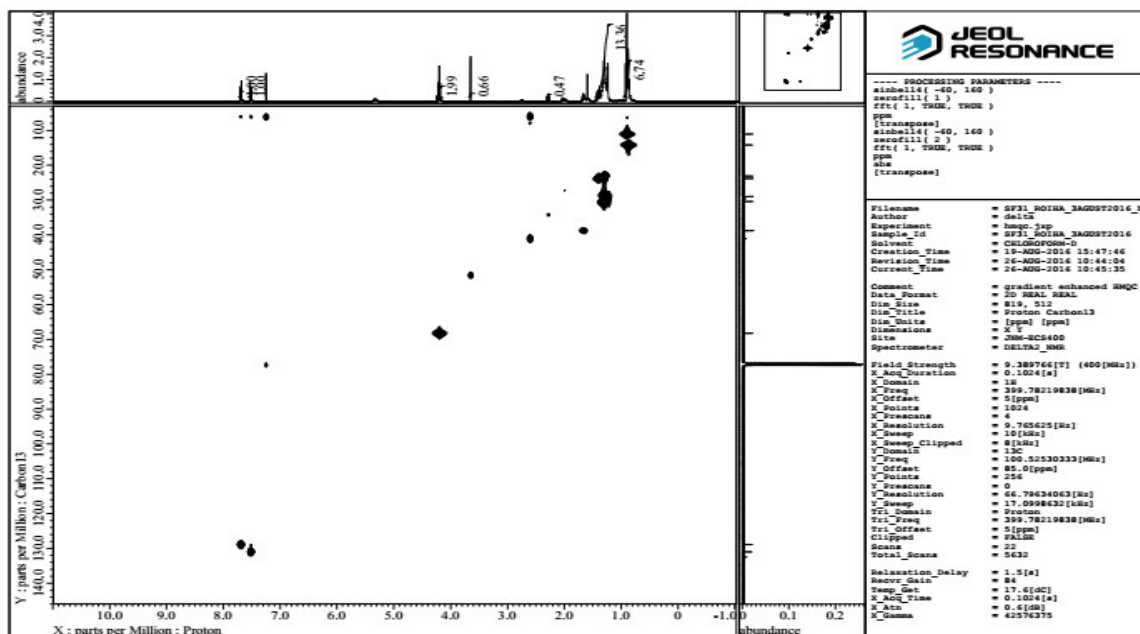


Figure S3. HMQC of compound 1.

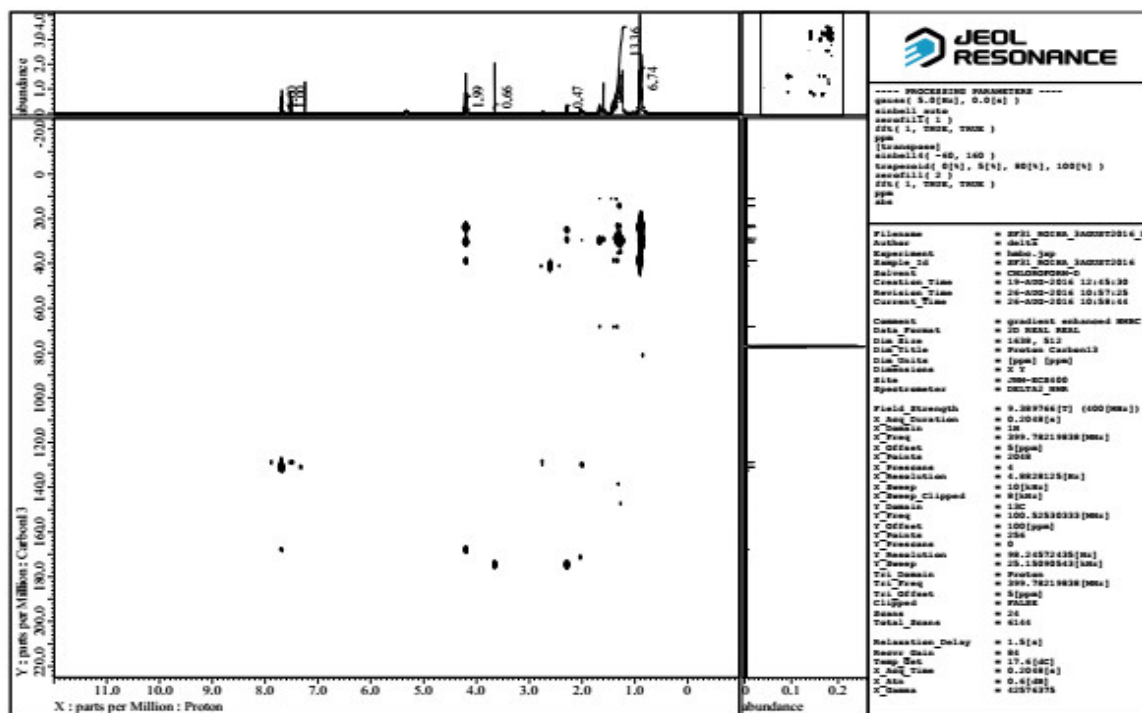
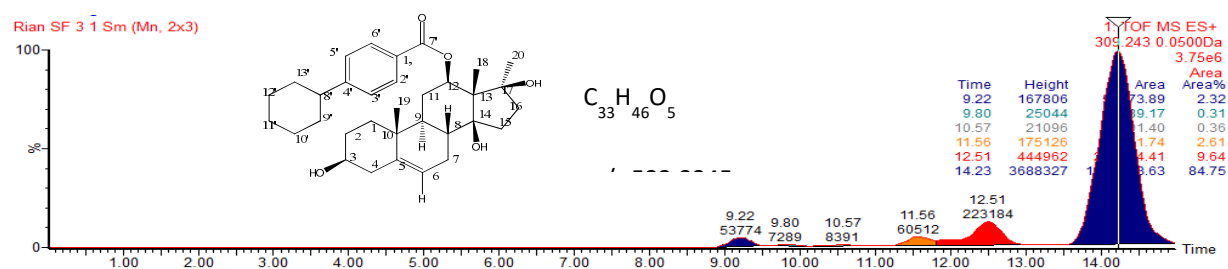
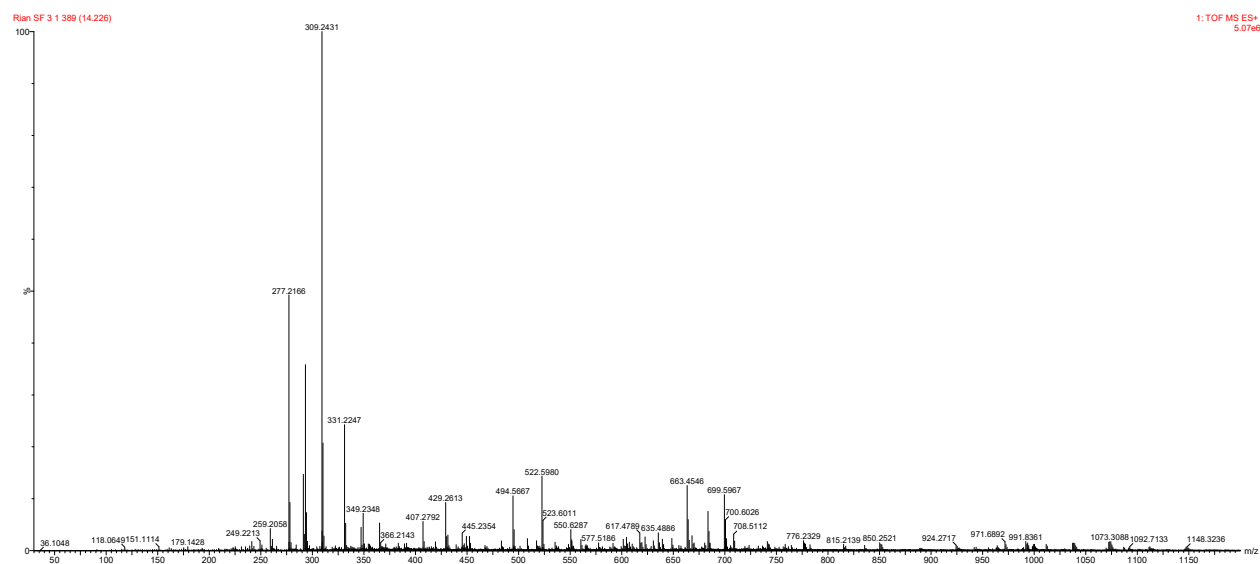


Figure S4. HMBC of compound 1.

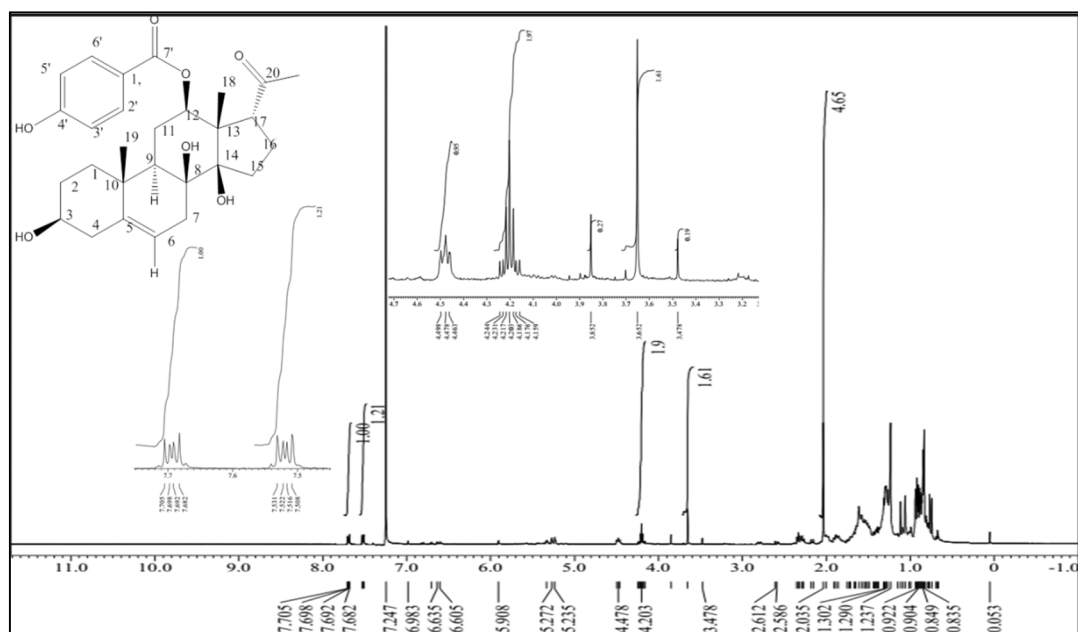
## Compound 1



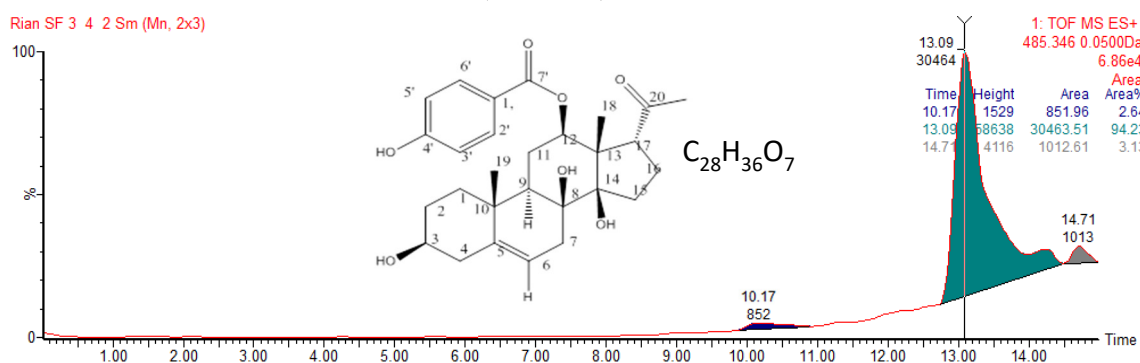
**Figure S5.** Profile of chromatogram UPLCMS / MS with stationary phase of Sunfire C18 2.2 x 50 mm column, and fixed mobile phase of ACN: H<sub>2</sub>O: formic acid, flow rate of 1 ml / min (a) isolate 3.1 root of *C. Gigantea* single peak chromatogram at retention time of 14.22 min identified as molecule with formula of C<sub>33</sub>H<sub>46</sub>O<sub>5</sub>, m/z 522, 3380.



**Figure S6.** Spectra m/z of compound 1.



**Figure S7.** HNMR Spectra of isolate 3.4.2 from the root part of *Calotropis gigantea*, 5 mg sample was dissolved in CDCL<sub>3</sub> with internal standard of TMS, analysis was using NMR JEOL ECS400 (400 MHz).



**Figure S8.** Profile of Chromatogram UPLC-MS / MS with stationary phase of Sunfire C18 2x 50 mm column, and mobile phase Gradient of ACN: H<sub>2</sub>O, flow rate 1 ml / min (a) isolate 3.4.2 from Roots of *Calotropis gigantea*, single peak at retention time of 13.09 min, identified as molecule with formula of C<sub>28</sub>H<sub>36</sub>O<sub>7</sub>, m/z 484.2460.



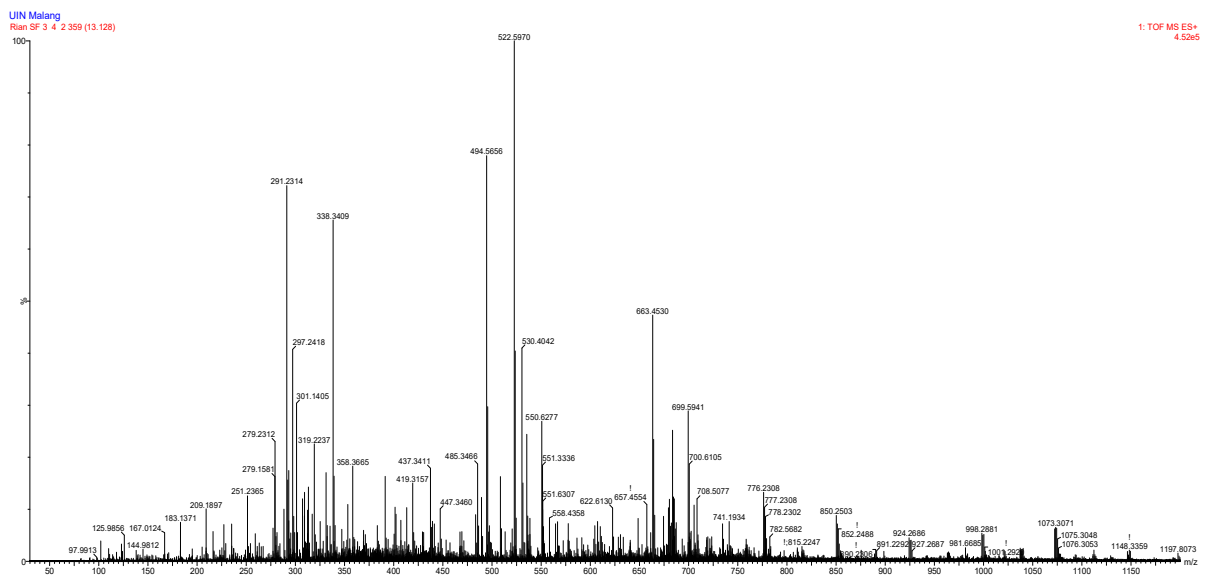


Figure S9. spectra m/z of compound 2.