The Marker Active Compound Identification of *Calotropis gigantea* Roots Extract as an Anticancer

Roihatul Mutiah, Sukardiman, Aty Widyawaruyanti

***Abstract*—** *Calotropis gigantiea* (L.) R. Br (Apocynaceae) commonly called as "Biduri" or "giant milk weed" is a well-known weed to many cultures for treating various disorders. Several studies reported that *C.gigantea* roots has anticancer activity. The main aim of this research was to isolate and identify an active marker compound of *C.gigantea* roots for quality control purpose of its extract in the development as anticancer natural product. The isolation methods was bioactivity guided column chromatography, TLC and HPLC. Evaluated anticancer activity of there substances using MTT assay methods. Identification structure active compound by UV, ^{1}HNMR, ^{13}CNMR, HMBC, HMQC spectral and other references. The result showed that the marker active compound was identical as Calotropin.

***Keywords*—** calotropin, *Calotropis gigantea*, marker active, anticancer.

I. INTRODUCTION

Calotropis gigantea is a plant that is widespread in Indonesia, the plant known as widuri. In previous studies have reported some anticancer effects of the roots of the plant are the roots of *Calotropis gigantea* ethanol extract could inhibit colon cancer cells widr in IC_{50} of 44,2 µg/ml (1). calotroposide K was reported inhibiting the growth of U373 glioblastoma (GBM) and PC-3 prostate cancer cell lines with IC_{50} (0.5 µg / ml and 0.7 µg / ml). calotroposid M was known inhibiting the growth of U373 glioblastoma (GBM) and PC-3 prostate cancer cell lines in IC_{50} 0.5 µg/ml and 0.7 µg/ml (2). The in vitro growth inhibitory activity of the n-BuOH fraction and compounds (Calotroposid H-N) was reported against A549 non-small cell lung cancer (NSCLC), U373 glioblastoma (GBM), and PC-3 prostate cancer cell lines (3). Antitumour activity of *Calotropis gigantea* root bark against Ehrlich ascites carcinoma in Swiss albino mice was reported. The result showed that a significant decrease in viable tumor cell count and a significant increase of life span in the ME (methanol extract) and CF (chloroform soluble fraction) treated group compared to untreated one. The life span of ME and CF treated animals was significantly ($P<0.05$) increased by 43.90% (20 mg ME/kg) and 57.07% (40 mg CF/kg). ME and CF broµght back the hematological parameter more or less normal level. ME and CF also restored the altered levels of serum alkaline phosphatase (SALP) and serum glutamate oxaloacetate transaminase (SGOT) (4). Based on these studies the roots of *Calotropis gigantea* an be developed into phytopharmaca products.

This study is one part of the process to develop roots (*Calotropis gigantea*) into phytopharmaca product used for the treatment of colon cancer. Phytopharmaca products can provide a consistent therapeutic activity if the raw material has a consistent composition or content. So that the assay of the constituents in the raw materials into one of the parameters in the process of standardization of raw materials. One approach that can be used to analyze the preparation of natural medicine is to establish the levels of constituents responsible for the activities fitofarmaka product or active marker (5).

The introduction and development of several techniques specific in vitro bioassay, chromatography, and spectroscopy techniques to make the screening process, the isolation and identification of the lead compound to be fast and precise. So for drµg discovery from natural materials can approach bioassay guided isolation (6).

In this study aims to isolate the marker compound of 70% ethanol extract of the roots (Calotropis gigantea) with the approach of bioassay guided isolation. To isolate a few action steps that liquid-liquid fractionation, column chromatography, TLC, HPLC. As for the identification of marker compounds used methods UV, HNMR, CNMR, HMBC and HMQC.

II. MATERIAL AND METHODS

A. Plant Material

Calotropis gigantea leaves were collected and identified in Lembaga Ilmu Pengetahuan Indonesia (LIPI) Purwodadi, East Java, Indonesia.

B. Materials for extraction, separation processes, isolation and identification of structures

Ethanol (technical-redistilled), n-hexane (technical-redistilled), ethyl acetate (technical-redistilled), chloroform pa (JT Backer), methanol (technical-redistilled), methanol pa (JT Backer), methanol pro HPLC (JT Backer), dichloromethane

Roihatul Mutiah is Doctoral Program, Faculty of Pharmacy, Airlangga University , Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia (phone: 031-5033710; fax: 031-5020514; e-mail: roihatulmutiah@gmail.com) .

Sukardiman is Departement of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia (e-mail: maman_ht@yahoo.com)

Aty Widyawaruyanti is Departement of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia (e-mail: aty_ww@yahoo.com) .

(technical-redistilled), pro HPLC acetonitrile (JT Backer), aquabidest, TLC plates silica gel 60 F254 (Merck 0.25 mm), silica gel 60 for column chromatography (Merck 0.063 to 0.200 mm), silica gel 60 G (Merck), CDCl3 (chloroform-D) pro NMR, tetramethylsilane pro NMR.

C. Cytotoxicity testing materials

Cell cultures of colon cancer WiDr collection of the Laboratory of Parasitology, Faculty of Medicine, aquabidest, sodium bicarbonate (Sigma) and hepes (Sigma), media RPMI, fetal bovine serum (FBS) 10% v/v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungison 0.5% v/v (Gibco), Phosphate Buffer Saline (PBS) 20% (Sigma); Dimethyl sulfoksid (DMSO); reagent MTT (3- (4,5-Dimetiltiazol-2-yl) - 2,5-difeniltetrazolium bromide) 5 mg in 1 ml of PBS (Sigma); Stopper: Sodium Dodecyl Sulphate (SDS) 10% in 0.01 N HCl (Merck), 96 well plate, pipette tips (1-1000 mL) sterile, 0.22 µm filter unit MIllex-GV (Millipore), trypan blue stain 0.25% (Gibco).

D. Research Instruments

Tools for the extraction, the process of separation, isolation and purification of active compounds: glassware, rotary, analytical balance, vacuum column, the column is open, chamber, vial, spray stains, capillary tube, fume hoods, TLC Visualiser (CAMAG), UV light 254 and 365 nm (CAMAG), High performance Liquid Chromatography (Shimadzu LC-06), RP column shimpack 4.6 x 250 mm, Prominanence DGU-20A5 degasser, Pump LC-6AD, shimpack column 4,6x250 mm, Prominence Diode Array Detector SPD-M20A. Tools to identify active compounds NMR Spectrometer JEOL radio wave frequency of 400 Hz (JNM-ECS400). Tools for testing cytotoxicity: CO2 incubator (New Bronswick, Galaxy 170 R), sentrifµge (Hermle Siemensstr-25 D-78 564), sonicator, Laminar Air Flow cabinet (Mascotte LH-S), a micropipette (soccorex), autoclave, hemocytometer, microscope inverted (Olympus CKX41-2), ELISA reader (Robonik).Tools for identification of the active ingredient and the active compound: densitometer (CAMAG), NMR Spectrometer JEOL radio wave frequency of 400 Hz (JNM-ECS400), ^{13}CNMR Joel resonance 400 Hz.

E. Extraction and isolation

One kilogram of *Calotropis gigatea* was extracted with 70% ethanol at 60°C, yielded 41.9 g of crude extract. It was fractionated gradually with dicloromethane, ethyl acetate and butanol to yield four fractions including dichloromethane fraction (F1=23.7 g), ethyl acetate fraction (F2 =3.2 g), butanol fraction (F3=6.1 g) and water fraction (F4=8.0 g). Fraction 2 (3.2) was separated by vacuum liquid chromatography with Stationary Phase: Silica gel 50g, column diameter :5 cm, Column height : 5 cm. Mobile phase :@40ml; Chloroform 100%; chloroform: methanol 95/5; chloroform: methanol 90/10, 80/20, 70/30, 60/40, 50/50, methanol 100%), yielded 7 major subfraction (SF1-SF7). Further separation of subfraction SF3 was conducted by open column chromatography with Stationary Phase: Silica gel 60, column diameter :1 cm, Column height :9. 5 cm. Mobile phase : n-heksan :EA (4:1), EA: Chlorofom (9:1), Chloroform: metanol (1:1), yielded 6 isolat (SF 3.1-SF3.6). Calotropin : white paste, UV 226 ^{1}H and ^{13}C NMR data are given in table 1.

F. HPLC Condition

HPLC analysis was conducted using ACN:H_2O (50:50-95-5) as mobile phase by Gradient elution, RP *shimpack* 4,6 x 250 mm column at flow rate of 0.7 ml/minute, column temperatureof 30°C, stop time of analysis at 60 minutes and detection wavelength set at 254nm and 385 nm. Gradient moble system : 0-5 min (ACN 50%:Water 50%); 5-40 min (ACN 95%:Water 5%); 40-60 min (ACN 95%:Water 5%) .

G. Cytotoxic assay

The human colon cancer WiDr cell lines were cultured in 96-well plates (Becton Dickinson Co., NJ., USA), and each well contained 5x10^3 cells. The culture cells were incubated in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 and 95% air for 24 hours. Cell confluence or crowding of cells in the plate was about 70-70%. After 24 hours incubation, culture medium was discarded. The cells were treated by *Calotropis gigantea* extract and fractions (treatment groups) or the vehicle (control group), and then incubated for 24 hours. The concentrations of the fraction were 1, 10, 100, 500 and 1000 µg/ml in DMEM. After incubation, the cells were incubated with 0.5 mg/ml MTT for 4 hours in 37°C. Viable cells react with MTT to produce purple formazan crystals. After 4 hours, the stopper 10% SDS (Sigma Co., St.louis, MO) in 0.01 N HCl (Merck) was added to dissolve the formazan crystal. The cells were then incubated for 24 hours in room temperature and protected from light. After incubation, the cells were shaken, and cells absorbance was measured by ELISA reader at λ 595 nm.

III. RESULT AND DISCUSSION

Calotropis gigantea root dry powder was extracted using ethanol 70%. In a preliminary test against WiDr colon cancer cells, it was found that 70% ethanol extract of the roots of Calotropis gigantea can inhibit cancer cell growth with IC50 value of 44.2 µg / ml. Further fractionation performed on a crude extract with a liquid-liquid partition method and the resulting four fractions: DCM fractions, fractions EA, butanol fraction and water fraction. The result of anti-cancer activity in the fourth of the faction respectively 0,367 µg / ml, 0.063 µg / ml, 0:18 µg / ml and 8493 µg / ml.

These results showed that the fraction etyl acetate has a higher anticancer activity than other fractions. Furthermore, EA fraction separated by vacuum column chromatography with silica gel 60 as the stationary phase and gradients Chloroform methanol as the mobile phase with increased polarity. Such separation has produced 7 sub-fractions: SF1-SF7. The test results anticancer activity sub-fractions: SF1

(203.67 µg / ml ± 2.3); SF2 (1940 µg / ml ± 9.5); SF3 (3.41 µg / ml ± 0089); SF4 (14:31 µg / ml ± 1.96); SF5 (360 µg / ml ± 2.74); SF6 (811.54 µg / ml ± 9.6); SF7 (248.95 µg / ml ± 2.85. Sub-fractions toxicity test results showed that SF3 has the highest anticancer activity compared with the other sub-fractions. So SF3 separated further to get active anticancer compound. The method used for the separation of active compounds in SF3 is open column chromatography with the stationary phase silica gel G 60 and the mobile phase n-hexane: EA (4: 1); EA: Chlorofom (9: 1); Chloroform: methanol (1: 1).

This separation results obtained 6 isolates (SF3.1-SF3.6). the results of TLC using stationary phase silica gel GF254 and the mobile phase n-hexane: etyl acetate (4: 1) it is known that there is a single spot on SF 3.1 (Rf = 0.89) which seemed obvious at UV 254, and light brown on the UV 366 after spray with 10% H2SO4 heating 105ºC. TLC results using RP-18 stationary phase and a mobile phase acetonitrile: methanol: water (3: 2: 1) gives a dark blue stain at Rf 0:52. Profile TLC extract, fractions, sub-fractions and isolates are presented in the figure below.

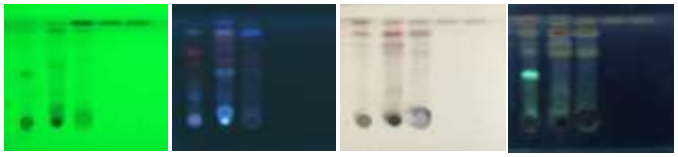

Fig.1 Profile TLC crude extract (S), etyl acetate fraction (F2), sub fraction 3 (SF3) and isolates calotropin (SF3.1). GF254 silica gel stationary phase and the mobile phase n-hexane: EA (4: 1). after eluasi and viewed under UV 254 nm (a); observed under UV 366 nm (b); after being sprayed with 10% H2SO4 and heated to 105ºC (c); after being sprayed, heated and viewed under UV 366 nm

Identification using 1H-NMR spectroscopy (Table 1) showed that the characteristic signal were benzoyl group at δ 7.716 (2H, d); δ 7.68 (1H, t); δ 7.52 (2H, t). signal one olefinic proton at δ 5.36 (1H, m), two protons hydroxyl group at δ 3.65 (2H, s), one proton hydroxyl group at δ 3.46 (1H, s), three methyl groups at δ 0.903 (9H, s), four methyl groups at δ 1.289 (12H, s), the methyl groups on δ1,59 (3H, s), two methylene groups at δ 2.23 (3H, t).

Identification using 13C NMR showed the presence of one carbon with ketone group at δ 167.8 ppm, carbon contained in benzene at δ 132.503 ppm; 130.997 ppm; 128.89 ppm; 128, 117 ppm. There is carbon with a methyl group on δ11.061 ppm; 14.179 ppm; 23.085 ppm; 23,800 ppm. There is a carbon by a methylene group at the δ 29 006 ppm, 30.47 ppm, 34 201 ppm; 38 778 ppm. There is a group methine carbon with the δ51.574 ppm and 68.231 ppm. 1H-NMR spectroscopy and 13C-NMR mentioned above have compatibility with spectroscopic data 1H-NMR and 13C-NMR has been reported by Wang et al (2008) and you at al (2013) (7), (8).

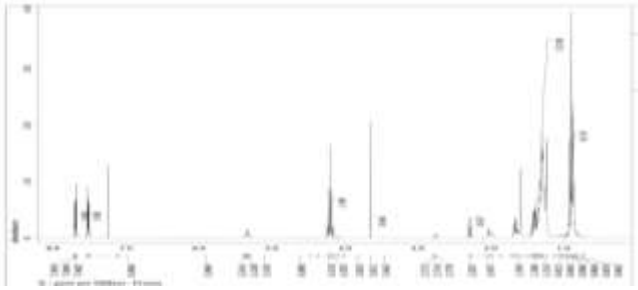

Fig. 2 The spectra of 1H-NMR spectroscopy of active marker compound isolated from *Calotropis gigantea*

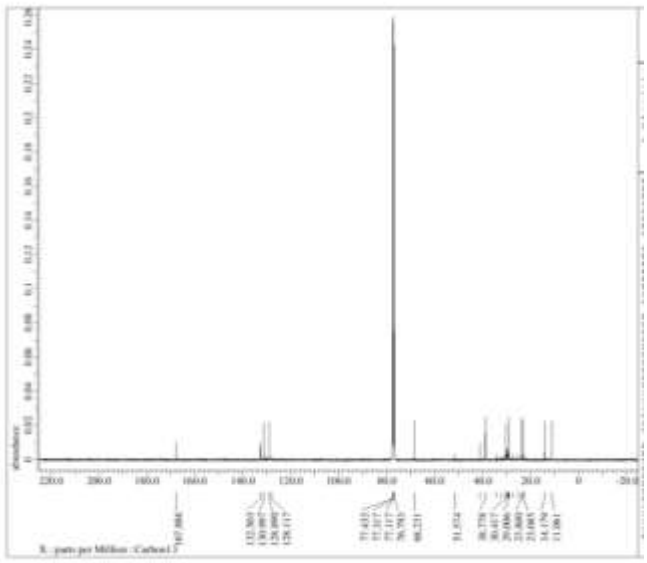

Fig. 3 The spectra of ^{13}C-NMR spectroscopy of active marker compound isolated from *Calotropis gigantea*

TABEL 1. THE 1H NMR AND 13C NMR (500 MHZ) DATA FOR MARKER COMPOUND

C	δ H (400 Hz)	δ C (400 HZ)	HMQC	HMBC
1	1.745 (1H, m), 1.11 (1H,m)	30.41	C11-H8	C14-H10
2	1.594, 1.30 (1H,m)	29.46	C12-H7	C2-H3/H5
3	3.65 (2H, s) , 3.45 (1H,s)	68.231	C1-H1	C3-H2
4	2.306 (1H, dd), 2.25 (1H, overlapped)		C2-H1	C3-H3
5	.		C3-H2	C3-H5/H7
6	5.361 (1H, m)		C4-H2	C4-H1
7	2.26, 1.984 (each 1H, m, J=0.47)	25.8	C5-H2	C4-H13
8	1,844 (1H,m)	34.2	C6-H2	C5-H2/H1
9	1.316 (1H, m)	38.77	C7-H2	C5/H5/H3/H7
10		29.79	C8-H3	C5-H8
11	2.001, 1.984 (1H,m, overlapped)	23.08	C10-H6	C5-H13
12	4.229 (1H, dd)			C6-H8
13		41.07		
14		51.57		C8-H1-H2
15	2.15, 1.98 (each 1H,m)	29.21		C4-H10
16	2.806 (1H,m), 1.88 (1H,m)	29.25		C7-H10
17		68.23		C8-H10
18	1.30 (3H,S)	11.06		
19	0.903 (9 H, m)	14.18		
20		167.89		
21	2.03 (H,m)	27.0		
1'		130.97		
2'	7.716 (1H,d)	128.89		
3'	7.52(1H,t)	128.117		
4'	7.68(1H,t)	132.503		
5'	7.52(1H,t)	128.117		
6'	7.716(1H,d)	128.89		
7'		167.886		

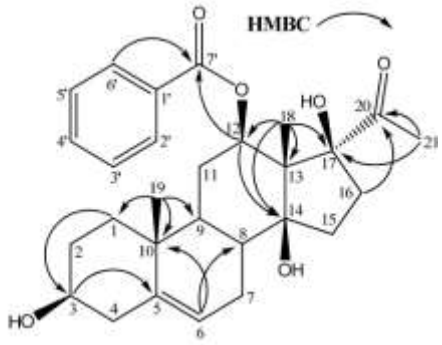

Fig. 4 The chemical structure of active marker compound isolated from *Calotropis gigantea*

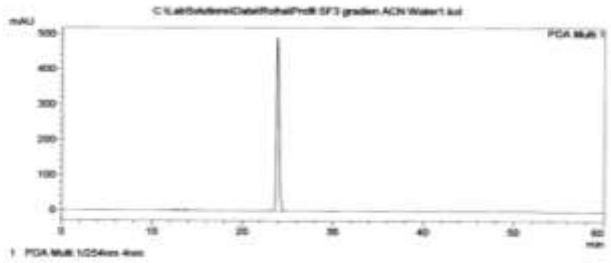

Fig. 5 Chromatogram marker compound obtained from ethanol extract of *Calotropis gigantea* roots

Based on data analysis using HPLC, HNMR, ^{13}C NMR, HMQC and HMBC and literature studies it can be determined that the SF 3.1 is identical with the compound Calotropon. The test results of the anticancer activity of SF 3.1 isolates in vitro against colon cancer cells WiDr shows IC_{50} 15.89 ± 1.36 µg / mL (Table 2). Under terms of the NCI, the SF 3.1 isolates showed effective anticancer colon. Based on the above data then isolate SF 3.1 can be used as an active marker.

IV. CONCLUSION

The result of this study showed that the active marker compound, Calotropin, can be used as a marker compound in standardization of ethanol extract of *Calotropis gigantea* roots as anticancer phytomedicine product. Further studies are needed to develop an analysis method of Calotropin for quality control and standardization purpose of ethanol extract of *Calotropis gigantea* roots.

.

ACKNOWLEDGMENT

We deeply thank the DP2M DIKTI (Directorate of Higher Education) Ministry of Education, Indonesia, for their financial support throµgh"HIBAH KERJASAMA PERGURUAN TINGGI (PEKERTI) Research Grant in 2014 that made this study possible.

.

REFERENCES

1. Muti R, Widyawaruyanti A, Zulaikah S. Comparison of Ethanol Extract from Roots , Leaves , and Flowers of. 2016;18:1–4.
2. Ibrahim SRM, Mohamed G a., Shaala L a., Banuls LMY, Van Goietsenoven G, Kiss R, et al. New ursane-type triterpenes from the root bark of Calotropis procera. Phytochem Lett [Internet]. Phytochemical Society of Europe; 2012 Sep [cited 2013 Nov 17];5(3):490–5. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1874390012000924
3. Ibrahim SRM, Mohamed G a., Shaala L a., Banuls LMY, Kiss R, Youssef DT a. Calotroposides H–N, new cytotoxic oxypregnane oligoglycosides from the root bark of Calotropis procera. Steroids [Internet]. Elsevier Inc.; 2015;96(January):63–72. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0039128X

1500032X
4. Habib MR, Karim MR. Evaluation of antitumour activity of Calotropis gigantea L. root bark against Ehrlich ascites carcinoma in Swiss albino mice. Asian Pac J Trop Med [Internet]. Hainan Medical College; 2011 Oct [cited 2013 Nov 17];4(10):786–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22014733
5. Folashade O, Omoregie H, Ochogu P. Standardization of herbal medicines - A review. 2012;4(March):101–12.
6. Sarker SD, Gray AI. Natural Products Isolation. second edi. Humana Press; 2006.
7. Wang Z-N, Wang M-Y, Mei W-L, Han Z, Dai H-F. A new cytotoxic pregnanone from Calotropis gigantea. Molecules [Internet]. 2008 Jan [cited 2013 Nov 15];13(12):3033–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19052526
8. You H, Lei M, Song W, Chen H, Meng Y, Guo D, et al. Cytotoxic cardenolides from the root bark of Calotropis gigantea. Steroids [Internet]. Elsevier Inc.; 2013;78(10):1029–34. Available from: http://dx.doi.org/10.1016/j.steroids.2013.06.002