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Original Article

CYTOTOXIC BIOACTIVE COMPOUNDS FROM *CALOTROPIS GIGANTEA* STEM BARK

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ABSTRACT

Objective: The present study deals with the cytotoxic activity of n-hexane and ethyl acetate extracts of *Calotropis gigantea* L. stem bark and its fractions such as A, B, C, D and E fractions on murine leukemia cell line P388.

Methods: The crude extracts of *C. gigantea* stem bark were prepared using n-hexane and ethyl acetate solvents. The plant extracts were subjected to vacuum liquid chromatography followed by TLC. According to the similarity of stain patterns, the fractions were combined. The extracts and its combined fractions were then subjected for the phytochemical test. Cytotoxic activity of those extracts and its combined fractions were tested using MTT assay. Fraction D was subjected to gravity column chromatography followed by TLC. Then, fractions A, B, and D2 were crystallized and subjected to GC-MS.

Results: The qualitative screening of n-hexane extract of *Calotropis gigantea* L. stem bark for secondary metabolites showed the presence of terpenoid, flavonoids, phenolics and coumarins. While the ethyl acetate extract contained phenolics, steroids, flavonoids, saponins and coumarins compounds. IC_{50} values for n-hexane extract and E fraction are 76.29 µg/ml and 18.48 µg/ml, respectively. In the ethyl acetate extract and C fraction obtained IC_{50} values 57.05 µg/ml and 52.58 µg/ml.

Conclusion: Cytotoxic activity from E fraction of n-hexane extract of *C. gigantea* stem bark is the most potent and containing flavonoids, phenolics and coumarins. The main components from several compounds of n-hexane extract of *C. gigantea* are germacrane-A, (-)-globulol, urs-12-ene and veridiflorol.

Keywords: *Calotropis gigantea*, Cytotoxic activity, IC_{50}, MTT assay

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INTRODUCTION

Tumors and cancers are classified as diseases those are potentially dangerous to human life. The World Health Organization (WHO) reported deaths from cancer by about 13% every year [1]. According to the International Agency for Research on Cancer (IARC) in 2008, worldwide, it was estimated that there were 12.7 million new cases, 7.6 million deaths; of these number, 56% of the cases and 64% of the deaths occured in the economically developing countries [2, 3]. The success of cancer therapy is still relatively low; this is due to limitations in the use of anticancer associated with safety because almost all anticancer not only kill the cancer cells but also cause damage and death in normal cells [4]. The problem is further complicated cancer because most cases are found at an advanced stage, the survival rate is low and costly to handle. Therefore it is necessary to do the discovery and development efforts of new cheaper anticancer.

One of the plants that attract attention is *C. gigantea.* The roots of this plant are used to treat gastric cancer. However, inventarization of these plants is still low so that the cytotoxic activity of *C. gigantea* in particular, which grows in the region of Aceh is not known yet. Previous studies conducted by Wang, *et al.* (2008) have isolated an anticancer compound pregnanon that is calotropon of ethanol extract of the roots of *C. gigantea*. *C. gigantea* plants have chemical constituents such as kardenolida, cardiac glycosides, flavonoids, pregnan, gigantisin and non-protein amino acids. During the screening of cytotoxic materials from tropical medicinal plants, the ethanol extract of the roots of *C. gigantea* showed cytotoxic activity against K562 chronic myelogenous leukemia, and human gastric cancer SGC-7901 *in vitro* using the MTT method with IC_{50} value of 9.7 µg/ml and 6.7 µg/ml. Bioassay-guided fractionation of the ethanol extract of *C. gigantea* roots produces a new compounds pregnanon, namely calotropon (1), with cardiac glycosides compounds (2). The structures of these compounds were determined by using 1D and 2D spectral data of NMR Spectroscopy. Compounds 1 and 2 showed significant cytotoxic activity against K562 cells and SGC-7901 [5].

The results of screening anticancer of ethyl acetate extracts of stem bark and leaves of *C. gigantea* with brine shrimp lethality test (BLST) method obtained a strong cytotoxic activity with LC_{50} values of each: 39.73 ppm and 35.86 ppm [6]. In the present study, further research has been carried out with fractionation of n-hexane and ethyl acetate extracts of the stem bark, phytochemical test and cytotoxicity test of combined fractions with the MTT method against P388 cells.

MATERIALS AND METHODS

Collection of plant material

Calotropis gigantea plant was collected from the wild growing population in Keudee Aceh village, Kecamatan Banda Sakti, Kota Lhokseumawe during March 2014. The plant was identified in the Herbarium Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Syiah Kuala University (Reg. No of the certificate: No. 218/UN11.1.28.1/DT/2014). The collected plant parts (stem barks) were separated from undesirable materials. The plant parts were sliced into small pieces and dried in open air under shade for one week.

The extraction process

The dried stem barks of *C. gigantea* were taken as much as±2 kg. Furthermore, the bark was macerated with n-hexane solvent (Merck, Germany) for 3x24 h in order to obtain the extract solution. Maceration was repeated until the extracts obtained were almost clear. Then, the process followed by filtering and the filtrate was concentrated by using a rotary vacuum evaporator (Heidolph, Germany) to produce concentrated n-hexane extract. Then, the weight of the extract was measured. After that the residual result from the first maceration was being macerated back with ethyl acetate solvent (Merck, Germany) for 3x24 h in order to obtain the

extract solution. Maceration was repeated until the extracts obtained were almost clear. Then, the process followed by filtering and the filtrate was concentrated by using a rotary vacuum evaporator (Heidolph, Germany) to produce concentrated ethyl acetate extract. Then, the weight of the extract was measured.

Fractionation of concentrated n-hexane and ethyl acetate extracts

Concentrated n-hexane and ethyl acetate extract eluent system specified with a suitable solvent comparison using a thin layer chromatography (TLC) plate (Merck, Germany). Subsequently, the n-hexane or ethyl acetate extract was drawn as much as 10 g and its components were separated using a vacuum liquid chromatography (VLC, glass part: Pyrex, USA, vacuum part: Buchi, Swizerland). Stationary phase in the form of silica gel, i. e silica gel 60 G (Merck, Germany) which is as much as 100 g. For the n-hexane extract using n-hexane and ethyl acetate, while for the ethyl acetate extract using dichloromethane (Merck, Germany) and methanol (Merck, Germany) as a mobile phase with gradient elution (based on the results of analysis by TLC). Accommodated fraction out of each 50 ml in erlenmeyer. The fractions were combined according to the similarity of stain patterns after eluted with eluent system obtained and also sprayed with reagent seric sulfate (Merck, Germany). This is called the combined fractions. Then, the concentrated extracts and combined fractions were tested to identify chemical constituents and MTT assay to P388 cells (Sigma Aldrich, USA).

Qualitative phytochemical screening

Chemical tests were performed on the n-hexane and ethyl acetate extracts of stem bark of *C. gigantea* and its combined fractions using standard procedure to identify the phytoconstituents [7].

3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) assay

The cytotoxicity assay was performed as the method that has been reported earlier [8,9]. The cells were harvested (2.5-3.0 x 104 cells/well) and inoculated on plates consisting of 96 wells. The cells were washed with PBS (phosphate buffered saline) and then inoculated cultured with and without sample (1 mg/ml of each n-hexane and ethyl acetate extracts from the stem bark of *C. gigantea* and also the combined fractions from n-hexane and ethyl acetate extracts). After 72 h of incubation, the medium aspirated. 10 ml solution of MTT (5 mg/ml in PBS pH 7.2) was added to each well and the plate was incubated for 4 h at 37 °C. After incubation, 100 ml of DMSO (<0.5%) was added to each well and then homogenized with a shaker for a color formazan stabilize for 15 min. For each sample, concentration variation was made starting from 0.1 µg/ml, 0.3 µg/ml, 1 µg/ml, 3 µg/ml, 10 µg/ml, 30 µg/ml, and 100 µg/ml, then, each concentration was measured three times (triplo). Absorbance reading was using microplate reader (BioRad, USA) at λ540 nm and the fraction of surviving cells was calculated. Artonin E (100 mg) was used as a reference drug. Inhibition of the cell for each sample was calculated by using OriginPro 8 software.

RESULTS AND DISCUSSION

Total yield extracts and its color

Extraction 2 kg stem bark of *C. gigantea* produced 1.97% n-hexane extract with greenish yellow color and 1.1% ethyl acetate extracts with brownish green respectively. The results of maceration can be seen in table 1.

Table 1: Results of maceration *C. gigantea* stem bark

Extract	Sample mass (g)	Extract color
n-hexane	38.47	Greenish Yellow
Ethyl acetate	22.00	Brownish Green

Vacuum liquid chromatography (VLC)

Fractionation of the extract was performed using VLC. Fractionation was performed using 100 g of silica gel 60G (stationary phase, Merck, Germany) and concentrated extracts of 7 g.

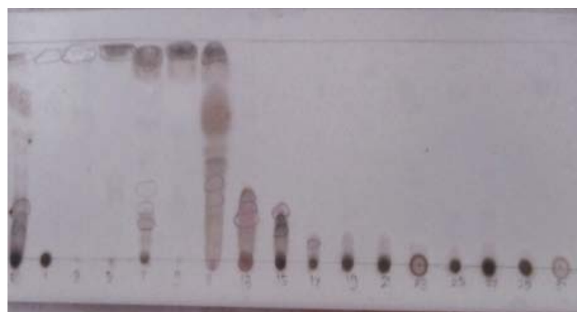

Fig. 1: The results of analysis from TLC Chromatogram using n-hexane extract, eluent system of n-hexane: ethyl acetate 9.5:0.5

Table 2: The combined fractions of n-hexane extract

Combined fraction	Combined fraction mass (g)	Fraction color
A	1.76	Light Yellow
B	13.37	Dark Yellow
C	2.45	Reddish Brown
D	1.40	Dark Green
E	0.84	Dark Green

Eluted using a gradient elution, to n-hexane extract used n-hexane and ethyl acetate, yielding 31 fractions (fig. 1), then the 31 fractions was held the TLC to see the patterns of the stains, and the fractions with the same stain patterns were combined to obtain combined fractions. The combined fractions of n-hexane extract can be seen in table 2.

The ethyl acetate extract was fractionated by gradient elution using a solvent of dichloromethane and methanol resulted in 14 fractions (fig. 2). The fractions on TLC were seen the stains pattern, and the same stain patterns were combined to obtain combined fractions. The combined ethyl acetate fractions can be seen in table 3.

Phytochemical analysis

Concentrated extracts and combined fractions of n-hexane and ethyl acetate extracts have been conducted a phytochemical test to see whether they contain secondary metabolites (tables 4 and 5).

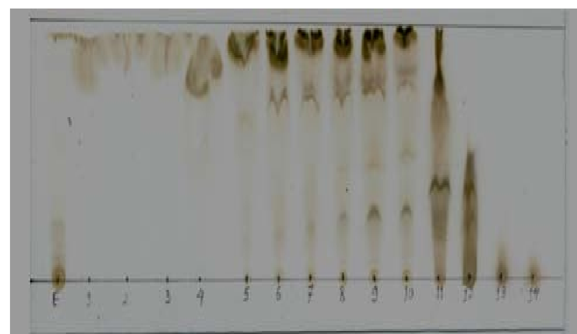

Fig. 2: The TLC Chromatogram from the results of fractionation of ethyl acetate extract using eluent system dichloromethane: methanol 98:2

Table 3: The combined fractions of ethyl acetate extract

Combined fraction	Combined fraction mass (g)	Fraction color
A	0.82	Light Yellow
B	0.35	Dark Green
C	1.72	Dark Green
D	6.55	Dark Green
E	4.55	Dark Green

Table 4: Test results of phytochemical n-hexane extract and its combined fractions

Secondary metabolites	n-hexane extract	Combined fractions				
		A	B	C	D	E
Alkaloid	-	-	-	-	-	-
Steroid	-	-	-	-	-	-
Terpenoid	+	+	+	+	+	-
Saponin	-	-	-	-	-	-
Flavonoid	+	-	-	+	+	+
Coumarin	+	-	-	-	+	+
Phenol	+	-	-	+	+	+

+= present; -= absent

Table 5: Test results of phytochemical ethyl acetate extract and its combined fractions

Secondary metabolites	Ethyl acetate extract	Combined fractions				
		A	B	C	D	E
Alkaloid	-	-	-	-	-	-
Steroid	+	-	+	+	+	-
Terpenoid	-	-	-	-	-	-
Saponin	+	-	-	+	+	+
Flavonoid	+	-	+	-	-	-
Coumarin	+	+	+	+	-	-
Phenol	+	-	+	-	-	-

+present,-absent

MTT assay

The results from each sample with the same concentration (n=3) were calculated to get the average values and the standard deviations by using Microsoft Office Excel 2013, which can be seen in table 6.

Table 6: Results of MTT assay of n-hexane and ethyl acetate extracts and its combined fractions (n=3)

Sample	IC_{50} (µg/ml)
n-hexane extract	76.29±19.56
Fraction E (n-hexane)	18.48±2.06
Ethyl acetate extract	57.05±4.70
Fraction C (ethyl acetate)	52.58±6.22
Artonin E (Control+)	0.67±0.01

From table 6 shows that the fraction E of n-hexane extract had the most powerful cytotoxic activity with IC_{50} value of 18.48 µg/ml. Based on the phytochemical test, the fraction contained flavonoids, coumarins, and phenolics compounds. Allegedly, the compound plays a role in contributing to the activity of the fraction E. Similarly, the fraction C of ethyl acetate extract, MTT assay results also indicate that the fraction C had good cytotoxic activity, with IC_{50} value of 52.58 µg/ml. The fractions contained saponins, coumarins, and steroids compounds. According to Caragay (1992), fourteen classes of phytochemical, the chemical components of plants, such as terpenoids, flavonoids, coumarins and phenolics, are known or believed to possess cancer-preventive properties [10]. Flavonoids also exhibit a wide range of biological activities like antimicrobial, anti-inflammatory, analgesic, anti-allergic, cytostatic and antioxidant properties [11].

Gravity column chromatography (GCC)

Seeing a little and inadequate amount of fraction E of n-hexane obtained, we used fraction D for further purification by using GCC, and the results of the phytochemical test showed that the fraction D contains the secondary metabolites close to the fraction E.

Fraction D as much as 0.9 g was separated using GCC using eluent n-hexane and ethyl acetate (gradient elution) as the mobile phase and silica gel G60 as the stationary phase. This separation resulted in 71 fractions. The fractions were analyzed by TLC using eluent n-hexane: ethyl acetate 9.5:0.5. TLC chromatogram from re-chromatography of fraction D results can be seen in fig. 3.

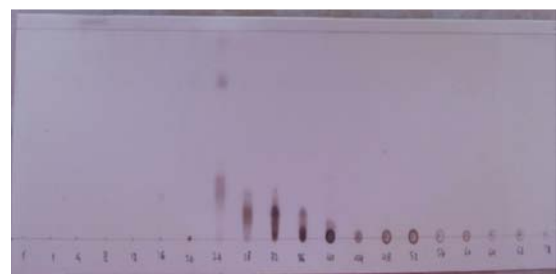

Fig. 3: TLC Chromatogram from re-chromatography result of fractions D

Based on fig. 3, fractions those have the same stain pattern combined to produce seven combined fractions namely D1, D2, D3, D4, D5, D6, and D7 (table 7). Fraction D2 was further purified by recrystallization.

Table 7: Fraction D of n-hexane extract gravity column chromatography results

Combined fractions	Combined fractions mass (g)
D1	0.01
D2	0.11
D3	0.08
D4	0.15
D5	0.21
D6	0.17
D7	0.03

Fraction A, fraction B and fraction D2 (fraction D re-chromatography results) were recrystallized using chloroform (Merck, Germany) and methanol (Merck, Germany) to remove impurities. Fraction A recrystallization produced as much as 1.07 g of a white solid (Compound A) with a melting point of 72-76 °C. This indicates that the compound was not pure. Fraction B was recrystallized three times produce as much as 0.38 g of a white solid (Compound B). Fraction D2 was recrystallized, and 0.03 g of an amorphous solid (Compound D2) was generated.

Gass chromatography-mass spectrometry (GC-MS)

Analysis of the components of the active fraction of *C. gigantea* stem n-hexane bark extract performed with a Shimadzu GCMS-QP2010S using Agilent % w column DB-1. The results of GC-MS characterization using crystalline, compound A showed that the crystals were still containing several compound components (table 8).

Table 8: Some of the compounds identified from the compound A of n-hexane extract of *C. gigantea* stem bark using GC-MS

Retention time	Compound name	Molecular formula	Molecular mass	% area	Similarity
29.194	Tetratetracontane	$C_{44}H_{90}$	618	10.76	97
33.272	Heneicosane	$C_{21}H_{44}$	296	16.42	96
50.134	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382	12.09	89
31.006	2-Hexyldecanol	$C_{16}H_{34}O_9$	242	5.27	90
34.184	Octacosanoic acid	$C_{29}H_{58}O_2$	438	7.41	93
40.602	Triacontanoic acid	$C_{31}H_{62}O_2$	466	10.55	94

The compounds contained in the compound A with a percentage of more than 5% that the retention time of 29.194 compounds identified a hydrocarbon compound that is tetratriacontane with an area of 10.76% and a molecular weight of 618 g/mol. Dwivedi *et al.* (2014) have identified a compound that is similar to the n-hexane extract of *C. procera* using GC-MS [12]. At the retention time, 33.272 identified a compound with molecular formula $C_{21}H_{44}$ heneicosane having a molecular weight of 296 with an area of 16.42%. While Mrunal, *et al.* (2013) have identified that heneicosane at a retention time of 33.19 had an area of 1.96% [13]. Tetracosanoic acid, methyl ester with an area of 12.09% at a retention time of 50.134 was identified. Subramanian, *et al.* (2012) have also identified tetracosanoic acid in *C. procera* [14]. Other compounds were identified that contained the compound A is 2-hexyldecanol, octacosanoic acid and triacontanoic acid.

The results of the analysis of chemical components of compound B with GC-MS showed that the compound is not purely with the main component sesquiterpene compounds, globulol (23%), then veridiflorol (17%) and urs-12-ene (16%).

Analysis of chemical components from compound D2 with GC-MS showed that the compound still contains several components with the main components of sesquiterpenes germacrene-A (44.04%), kauren-19-yl-acetate (17.4%), globulol (18.4%), and urs-12-ene (8.9%). While the analysis of fraction C, the ethyl acetate extract active fraction of *C. gigantea* stem bark, was performed with GC-MS, showed that the compound was not purely with the main component sesquiterpene compounds, globulol (31.41%), and urs-12-ene (26.25 %).

The structure of some of these compounds are shown in fig. 4. Further research is still needed for purification and determination of the active compound structure in spectroscopy which includes IR, NMR and Mass Spectrometry.

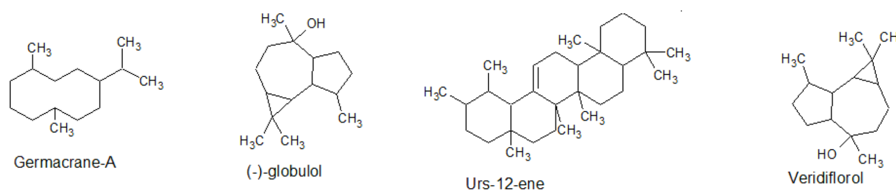

Fig. 4: Structure of several compounds have been isolated from the stem bark of plants of *C. gigantea* L.

CONCLUSION

The results of MTT assay fraction E of n-hexane extract was 18.48 µg/ml, while fraction C of ethyl acetate extract was 52.58 µg/ml.

The results of GC-MS analysis from fraction A of n-hexane extract obtained several compounds, which were tetratriacontane, heneicosane, tetracosanoic acid, methyl ester, 2-hexyldecanol, octacosanoic acid, triacontanoic acid; fraction B of n-hexane extract contain several components, such as globulol, veridiflorol, and urs-12-ene, fraction D2 of n-hexane extract contains germacrane-A, kauren-19-yl-asetat, globulol, and urs-12-ene. While fraction C of ethyl acetate extract contains globulol, and urs-12-ene.

Further study is required to purification the lead compound responsible for this activity and to investigate cytotoxic activity to P388 cell lines for the development of the new anticancer drug.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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