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Antiproliferative Activity of Triterpenoid and Steroid Compounds from Ethyl Acetate Extract of *Calotropis gigantea* Root Bark against P388 Murine Leukemia Cell Lines

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Abstract: *Calotropis gigantea* has been known to produce bioactive secondary metabolites with antiproliferative activities against cancer cells. Herein, we extracted the secondary metabolites using ethyl acetate from its root bark and further tested its antiproliferative activities against P388 murine leukemia cell lines. The subfractions from the ethyl acetate extract was obtained from Vacuum Liquid Column Chromatography (VLCC), and followed by Gravity Column Chromatography (GCC). The subfraction C_2 and D_1 were identified to contain triterpenoids and steroids with the most potent cytotoxicity against Brine Shrimp Lethality Test (BSLT). A 3-(4,5-dimethylthiazol-2-yl) -2-5 diphenyl tetrazolium bromide (MTT) assay suggested that ethyl acetate extract has the highest antiproliferative activities against P388 murine leukemia cell lines (IC_{50} = 21.79 µg/mL), as opposed to subfraction C_2 (IC_{50} = 50.64 µg/mL) and subfraction D_1 (IC_{50} = 49.33 µg/mL). The compound identified in subfraction C_2 and D_1 are taraxerol acetate and calotropone, respectively. Though taraxerol acetate and calotropone were active in inhibiting the leukemic cell lines, their IC_{50}s were lower than the ethyl acetate extract, which is probably due to the synergism of the secondary metabolites.

Keywords: leukemia; calotropone; taraxerol acetate; anticancer; *Calotropis gigantea*

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1. Introduction

Cancer is one of the most feared diseases in modern life and its treatment is usually carried out by chemotherapy, radiation therapy, surgery and immunotherapy [1,2]. Those treatments result in side effects, which eventually lead the researcher to look for alternative cancer treatments using natural compounds with high bioactivities. Furthermore, it is expected that the plant-derived compound may yield a lower risk of side effects in comparison with synthetic compounds [3]. One of the plants that is famous for its bioactive compounds is *Calotropis gigantea* [4,5]. This plant comprises chemical compounds such as alkaloids, resins, phenols, amyrin, sitosterol, isogiganterol, giganterol, flavonol glycosides, naphthalene, triterpenoids, tannins, saponins, sterols and steroids [6]. It also includes calotropone, an emerging bioactive compound with anticancer properties that has been extracted from *C. gigantea* root using an ethanol solvent [7].

Ethyl acetate extract of *C. gigantea* root has been reported to contain saponins, triterpenoids and coumarins [8]. The existence of triterpenoids such as calotropursenyl acetate and calotropfriedelenyl acetate could be shown from the isolation of root bark of *C. procera*; a species from the same family as *C. gigantea* [9]. Furthermore, one type of triterpenoid—

lupeol—could be found in the latex of *C. gigantea*, which has been reported to exhibit antitumor, anti-inflammatory and other beneficial activities [10].

Our recent study revealed that the methanol ethyl acetate, and n-hexane extracts from *C. gigantea* root bark, are highly cytotoxic against *Artemia salina* [11]. The highest median lethal concentration (LC_{50}) value of the aforementioned study reached 36.79 µg/mL for the ethyl acetate extracts. Next, this root bark ethyl acetate extract of *C. gigantea* was isolated and grouped into five combined fractions which have the same stain pattern. The two most potent of these combined fractions have cytotoxic activity against *A. salina* with an LC_{50} of 20.3 µg/mL and 18.9 µg/mL [11]. A study had successfully isolated α-taraxerol and β-sitosterol acetates from *C. gigantea* root bark showing cytotoxic activities against *A. salina* with LC_{50} values of 29.56 and 23.61 µg/L, respectively [12].

Despite its bioactive potential, only a few studies have reported on the use of extracts from *C. gigantea* root bark as an anticancer agent. The petroleum ether and chloroform extracts from *C. gigantea* root bark have been suggested to possess anti-tumor activities against Ehrlich ascites carcinoma in Swiss albino mice [13]. Pregnanone compounds, identified in 2008 [14], were reported to be active against chronic myelogenous leukemia K562 and human gastric cancer SGC-7901 cell lines. Herein, we have investigated the ethyl acetate extract from root bark of *C. gigantea*, which has never been reported for its activities against cancer cell lines. Triterpenoids and steroids were expected to be isolated from the extract. Furthermore, we determined each of the compound's structures whose potential anticancer activities are the highest against P388 murine leukemic cell lines. A natural compound—Artonin E—Was employed as the positive control because of its high activity against various cancers [15,16]. Moreover, artonin E has been used as the standardized positive control in the Research Center for Bioscience and Biotechnology, Institut Teknologi Bandung, Bandung, Indonesia.

2. Materials and Methods

2.1. Materials

The root bark of *C. gigantea* was collected from Musafir Area, Alue Naga Village, Syiah Kuala District, Banda Aceh, Indonesia during July–September, 2017. Identification of *C. gigantea* was held on 2 October 2017 at the Laboratory of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia (No. 943/UN11.1.28.1/DT/2017).

Solvents used in this study were methanol 95%, n-hexane, ethyl acetate, dichloromethane, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and sodium dodecyl sulphate (SDS). All chemicals used were analytical grade and purchased from Merck (Selangor, Malaysia). Silica gel GF_{254} for the Vacuum Liquid Column Chromatography (VLCC), silica gel G60 70–230 mesh ASTM for Gravity Column Chromatography (GCC) and Thin Layer Chromatography (TLC) plate were also purchased from Merck (Selangor, Malaysia).

Artemia salina (brine shrimp) eggs were purchased from Aquatic Animal Research Center, Universitas Syiah Kuala, Indonesia and used in the Brine Shrimp Lethality Test (BSLT). P388 cell lines were obtained from the collection of Research Center for Bioscience and Biotechnology, Institut Teknologi Bandung, Indonesia and used in the 5 3-(4,5-dimethylthiazol-2-yl) -2-5 diphenyl tetrazolium bromide (MTT) assay. The positive control, artonin E, was purchased from Natural Product Chemistry Laboratory, Institut Teknologi Bandung, Indonesia.

2.2. Extraction of C. gigantea Root Bark

The procedure followed the reported study with minor modifications [11]. Fresh *C. gigantea* root bark (9.7 kg) was washed with distilled water, cut into small pieces, and dried. The sample was then made into a powder using a crusher (Miyako, Jakarta Barat, Indonesia). The procedure was continued with maceration using methanol 95% for 3 × 24 h (3:7 kg/L). Next, macerate was filtered using filter paper and concentrated using a vacuum rotary evaporator (Heidolph, Schwabach, Germany) to acquire concentrated methanolic

extract. The extract was firstly suspended in distilled water and partitioned using n-hexane, ethyl acetate and methanol solvents, respectively. Afterwards, each extract was reconcentrated using a vacuum rotary evaporator (30–35 °C; 200 rpm).

2.3. Selection of the Combined Fractions Based on Their Cytotoxicity

Concentrated ethyl acetate extract (7 g) was fractionated using the VLCC with 100 g silica gel GF_{254} as the stationary phase. The extract was eluted using gradient elution of n-hexane:ethylacetate, and was followed by ethylacetate:methanol. The outgoing fraction was stored in an Erlenmeyer 100 mL until it reached the maximum volume marker, then concentrated. The procedure was followed by TLC plate (stationary phase: alumina) using eluent n-hexane:ethyl acetate system (7:3). Fractions with the same stain pattern were combined and concentrated to produce a series of combined fractions, namely A, B, C, D and E, which later were phytochemically tested following the suggestions of previous literature [11]. To identify which combined fraction had the highest cytotoxicity, BSLT was conducted against *Artemia salina* [17]. Each combined fraction of C and D exhibited the highest cytotoxicity and were thus used for further investigations. Fraction C was eluted with ethylacetate 100% and concentrated to obtain 0.3 g solid. Fraction D was eluted with ethylacetate:methanol (9:1) and was concentrated, yielding 2.7 g solid.

2.4. Isolation of Triterpenoids and Steroids

The isolation procedures were in accordance with the reported studies [12]. To obtain the triterpenoids, fraction C was chromatographed using a GCC method with silica gel G60 70–230 mesh ASTM and n-hexane:ethylacetate (7:3). The purer fractions were then recrystallized using a combination of n-hexane:dichloromethane and dichloromethane:methanol solvents with a gradient elution. From this GCC process, 20 subfractions ($C_{1–20}$) of combined fraction C were obtained and analyzed by TLC., of which a subfraction C_2 with a yellowish solid form was taken for phytochemical testing; triterpenoids were positive. This revealed that the subfraction containing triterpenoids had a Retention factor (Rf) of 0.62. Purification was carried out by recrystallization by using a methanol:dichloromethane solvent mixture, yielding 9 mg shiny yellowish crystals.

As for the steroids, a similar GCC method was employed against fraction D with gradient eluents of n-hexane:dichloromethane, and were followed by dichloromethane:methanol. The re-chromatography process of fraction D produced 40 subfractions (D_{1-40}) with different stain patterns. Subfraction D_1 was eluted with dichloromethane 100% and concentrated to obtained 120 mg solid. Subfraction D_1 formed an amorphous solid and was then reanalyzed by TLC using the chloroform:methanol eluent system (9.5:0.5). The phytochemical tests revealed that subfraction D_1 contains secondary metabolites of steroids, and then it was recrystallized using methanol:chloroform solvents to produce 50 mg yellowish-white solids.

2.5. 3-(4,5-Dimethylthiazol-2-yl) -2-5 Diphenyl Tetrazolium Bromide (MTT) Assay

The P388 cell lines at an initial cell density of approximately 2.5–3.0 × 104 cells/well were harvested and inoculated into 96-well plates. After 24 h of incubation for cell attachment and growth, the cells were washed with PBS and then inoculated and cultured with and without samples (1 mg/mL each extract of n-hexane, ethyl acetate and methanol from the root bark of *C. gigantea* and also the subfractions C_2 and D_1 of *C. gigantea* root bark ethyl acetate extract). For each sample, varying concentrations of samples were made by dissolving the samples in DMSO at the required concentrations from 0.1, 0.3, 1, 3, 10, 30, and 100 µg/mL and were added into the wells. The control well received only DMSO. After 72 h of incubation, the medium was aspirated and 10 µL of MTT solution (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 µL of DMSO (<0.5%) was added to each well and then homogenized with shakers for formazan stable colors for 15 min, at which point the MTT-stop solution containing SDS was added and another 24 h incubation was conducted. The absorbance was

read by using an ELISA reader at λ 540 nm and the surviving cell fraction was calculated. All of these testing processes were carried out three times. The number of cells that grew was proportional to the amount of formazan formed. Artonin E (100 mg) was used as a positive control. OriginPro 8 software (Northampton, MA, USA) was used to calculate cell inhibition for each sample. The half-maximal inhibitory concentration (IC_{50}) value was the concentration required for 50% growth inhibition.

2.6. Structure Characterization

The isolated compounds of subfraction C_2 were then characterized using Fourier Transform Infrared (FTIR) spectrometry (Shimadzu FT-IR 8400, Kyoto, Japan) and mass spectrometry (Shimadzu GCMS-QP 2010 Ultra, Kyoto, Japan). Characterization using FTIR spectrum was used to determine various types of functional groups contained in a compound. Small absorption ranges can be used to determine each type of bond (wavenumber range: 4000–400 cm^{-1}) [17]. Subfraction C_2 was also characterized with mass spectrometry to identify the contained compounds [18]. For subfraction D_1, the isolated compounds were characterized using FTIR spectrometry (Shimadzu FT-IR 8400, Kyoto, Japan) and Hydrogen Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy (JEOL ECA 500 Tokyo, Japan). Subfraction C_2 was only characterized with mass spectrometry because the number of the obtained sample was not adequate for ^{1}H-NMR.

3. Results

3.1. Extraction Yield and Phytochemical Properties

The methanol extract from maceration was concentrated with a vacuum rotary evaporator and produced concentrated methanol extract of 160.25 g (1.65% *w*/*w*). From the partitioning the methanolic extract from *C. gigantea* root bark, the concentrated extracts obtained were 20.47 g from n-hexane, 73.60 g—ethyl acetate and 66.18 g—methanol solvents.

The results of the phytochemical tests on the ethyl acetate extract from *C. gigantea* root bark reveal the content of secondary metabolites (such as steroids, triterpenoids, saponins, phenols and coumarin). The positive presence of steroids and triterpenoids in the sample after reacting using a Liebermann-Burchard reagent was indicated by changes in color, to green and red, respectively. The positive test result of saponin was characterized by the foam formation in the sample after it was shaken. The presence of phenol was characterized by the formation of a blue color. The coumarin presence was characterized by a greenish-yellow fluorescence color under a UV lamp [19].

3.2. Fractions and Their Cytotoxicity against Brine Shrimp Lethality Test

After the VLCC process, the fractions obtained were 26 fractions which were then analyzed by TLC. Those with the same stain pattern were combined, so that five groups of fractions were obtained, namely combined fractions A, B, C, D, and E. The same stain pattern was predicted to have the same secondary metabolite compounds. Each group of fractions was weighed and its components were analyzed using TLC.

A preliminary study using BSLT to examine the cytotoxic activity against A. salina showed that fractions A, B, C, D and E have LC_{50} of 593.8, 595.8, 20.3, 18.9 and 31.6 µg/mL respectively. Fractions C and D have the most potent cytotoxic activity; hence, they were selected to be further isolated by GCC, tested by MTT assay, and were elucidated for their structures [17].

3.3. MTT Assay of the Extracts from C. gigantea Root Bark

Table 1 shows that the ethyl acetate extract has an IC_{50} of 21.79 µg/mL, which means that it is more potent than its isolated compounds (Subfraction C_2 and D_1) and other extracts (n-hexane and methanol extracts). Ethyl acetate extract contains steroid, terpenoid, coumarin, saponin and phenol compounds. These five classes of compounds synergistically contribute to an increase in the antiproliferative activity of the P388 cell lines.

Table 1. The result of an MTT assay of n-hexane, ethyl acetate, methanol extracts and subfractions C_2 and D_1 (n = 3).

Sample	IC_{50} (μg/mL)
N-hexane extract	49.02 ± 0.06
Ethyl acetate extract	21.79 ± 0.03
Methanol extract	68.45 ± 0.02
Subfraction C_2 (ethyl acetate)	50.64 ± 0.03
Subfraction D_1 (ethyl acetate)	49.33 ± 0.01
Artonin E (positive control)	0.74 ± 0.27

3.4. Structure Characterization of Subfraction C_2 and D_1

Table 2 presents the results of FTIR analysis on subfraction C_2 and D_1. In subfraction C_2 FTIR spectral data, the absorption at wavenumber 2920 cm^{-1} was observed to be assigned to C-H moieties, and 1701 cm^{-1}—for C = O moieties. In addition, C=C at wavenumber 1462 cm^{-1} and C-O (ester) group at 1190 cm^{-1} were shown. As for subfraction D_1, the presence of O-H moieties was indicated by the vibrational band appearance at 3007 cm^{-1}. Furthermore, spectral bands observed at 2934, 1715 and 1057 cm^{-1} were assigned for C-H (alkane), C=O and C-O stretching vibrations.

Table 2. FTIR data from subfractions C_2 and D_1.

Functional Group	Wavenumber (cm^{-1})		
	Subfraction C_2	**Subfraction D_1**	**Ref. [20]**
O-H	Not detected	3007	3000–3700
C-H (alkane)	2920	2934	2800–3000
C=O	1701	1715	1700–1725
C=C	1462	Not detected	1400–1600
C-O (ester)	1190	1057	1050–1260

Mass spectrometry was carried out on subfraction C_2, where the results were revealed in Figure 1. The data of the spectral peak appearances from mass spectrometry, based on the compound's fragmentations, are presented in Table 3. The detected subfraction C_2 has peaks at m/z 467, 457, 410, 357, 344, 325, 284, 257 and 200, which are consistent to the reported MS profile of taraxerol acetate ($C_{32}H_{52}O_2$, molecular weight = 468.76 g/mol) [21]. The fragmentation pattern of taraxerol acetate compounds can be seen in the previously published report [21]. The structure was confirmed by the absence of hydroxyl group and the presence of C-O esters and C=C double bound in the FTIR spectrum (Table 2).

As for the compound elucidation of subfraction D_1, ^{1}H-NMR was employed, where its spectrum has been presented (Figure 2). The presence of five aromatic protons were observed at δ: 7.94; 7.44 and 7.55 μg/mL were observed in the ^{1}H-NMR spectrum, which are typical for calotropone (Table 4). FTIR data of D1 (Table 2) also confirms the presence of aromatic uptake at wavenumber around 1500 cm^{-1}. Spectral peak at δ: 5.40 μg/mL (1H, m) was assigned to the olefinic proton. The appearance of three high field methyl singlets could be observed at δ 1.39, 0.96 and 2.08 μg/mL. These chemical shift values of the isolated compound have similarities to that of reported calotropone [14].

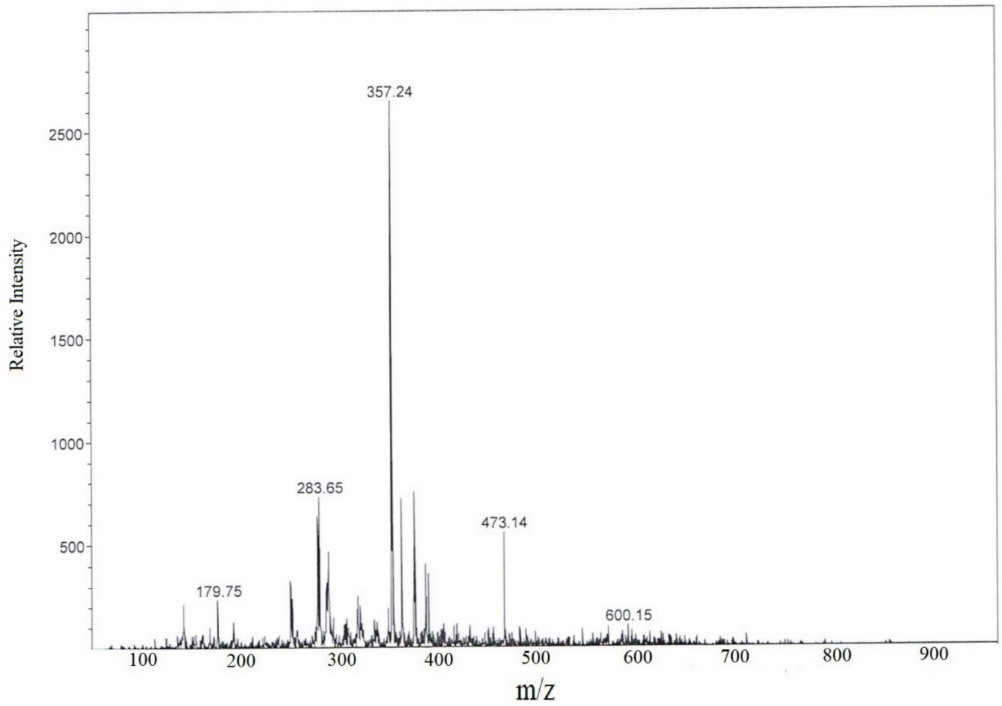

Figure 1. Mass spectrometry spectrum of subfraction C_2.

Table 3. Data on taraxerol acetate fragment ion data.

Fragment ion (*m*/*z*)	
Subfraction D_1	**Ref. [21]**
467	468
457	453
410	409
397	-
383	-
370	-
357	359
344	344
325	329
-	269
284	284
257	257
200	204
-	189
179	-
127	-
-	121

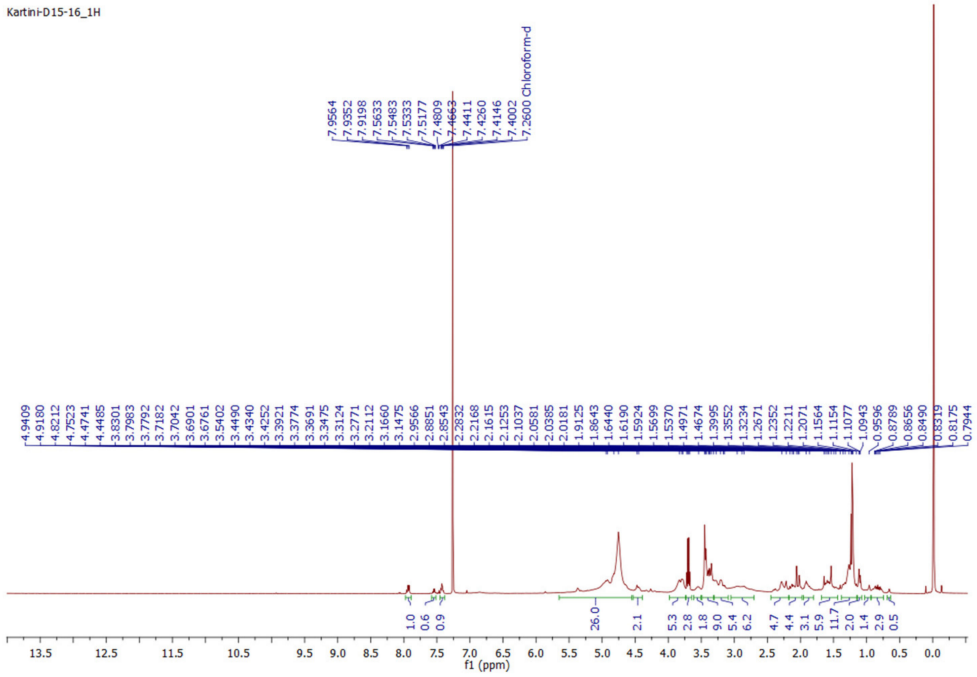

Figure 2. ^{1}H-NMR spectrum of subfraction D_1.

Table 4. Data of ^{1}H-NMR spectrum of subfraction D1 and reference of calotropone [14].

Position	δH (μg/mL)	
	This Study ($CDCl_3$, 500 MHz)	**Ref. [14] ($CDCl_3$, 400 MHz)**
1	1.74 (1H, m, H-1a),	1.75 (1H, m, H-1a),
2	1.12 (1H, m, H-1b)	1.13 (1H, m, H-1b)
3	1.82, 1.49 (each 1H, m)	1.81, 1.46 (each 1H, m)
4	3.54 (1H, m)	3.53 (1H, m)
5	2.31 (1H, m),	2.33 (1H, dd, 12.8, 3.6 Hz)
6	2.22 (1H, m, overlapped)	2.25 (1H, m, overlapped)
7	NA	NA
8	5.40 (1H, m)	5.41 (1H, m)
9	2.16, 1.92 (each 1H, m)	2.20, 1.91 (each 1H, m)
10	1.81 (1H, m)	1.80 (1H, m)
11	1.32 (1H, m)	1.32 (1H, m)
12	NA	NA
13	2.08, 1.47 (each 1H, m, overlapped)	2.06, 1.45 (each 1H, m, overlapped)
14	4.82 (1H, m)	4.80 (1H, dd, 11.3, 4.5 Hz)
15	NA	NA
16	NA	NA
17	2.13 (1H, m, H-15a),	2.12 (1H, m, H-15a),
18	1.94 (1H, m, H-15b)	1.92 (1H, m, H-15b)
19	2.89 (1H, m, H-16a), 1.89 (1H, m, H-16b)	2.90 (1H, m, H-16a), 1.88 (1H, m, H-16b)
20	NA	NA
21	1.39 (3H, s)	1.41 (3H, s)
1′	0,96 (3H, s)	0,98 (3H, s)
2′	NA	NA
3′	2.08 (3H, overlapped)	2.06 (3H, overlapped)
4′	NA	NA
5′	7.94 (1H, d, 7.3 Hz)	7.93 (1H, d, 7.5 Hz)
6′	7.44 (1H, t, 7.3 Hz)	7.43 (1H, t, 7.5 Hz)
7′	7.55 (1H, t, 7.5 Hz)	7.56 (1H, t, 7.5 Hz)
1	7.44 (1H, t, 7.3 Hz)	7.43 (1H, t, 7.5 Hz)
2	7.94 (1H, d, 7.7 Hz)	7.93 (1H, d, 7.5 Hz)
3	NA	NA

NA: not applicable, determined by ^{13}C-NMR.

4. Discussion

Calotropis gigantea from Aceh grows wildly like weeds with very strong supporting roots in the Alue Naga area; ±100–500 m from the shoreline and ±0.8 m above sea level. Some classes of chemical components of plants, such as triterpenoids, phenolic and coumarin, are believed to have cancer prevention properties [22]. In our study, five groups of secondary metabolites were obtained. Based on the phytochemical tests, the ethyl acetate extract was revealed to contain steroids, saponins, triterpenoids, phenols and coumarins. In the other study in India, the phytochemical analysis of the extracts from *C. gigantea* roots revealed the presence of saponins, triterpenoids and steroids [23]. Varied results obtained from the studies could be associated with the different geographical locations in which the plants grow. It is known that the topography and climate of the environment where the plants grow and develop could affect the production of their secondary metabolites [24].

Saponins have been reported for their antiproliferative effects by regulating the immune system [25–27]. Paris saponin had shown an inhibition effect towards cervical cancer and is a positive regulator of the immune system in tumor-bearing mice [25]. Furthermore, Paris saponin was revealed to have an inhibitory effect, significantly against U14 cells in vitro [26]. A mixture of monodesmoside saponins had been reported to be highly cytotoxic against colon and P388 cancer cell lines [27].

Along with saponins, coumarins have been reported in many different researches for having cytotoxic activities. One of the coumarin compounds obtained by multi-step synthesis of hydroxy benzophenones had shown excellent antiproliferative potential against murine EAC and dense DL tumors by triggering anti-angiogenesis and apoptosis [28]. A natural coumarin, osthole, showed inhibition in vitro towards Hepatocellular Cell Carcinoma (HCC) proliferation. In the G2 phase, it also causes cell accumulation and induces apoptosis. This coumarin can significantly suppress the growth of HCC tumors in vivo [29]. Other studies had shown that the coumarin metabolite, 4-hydroxycoumarin, inhibits cell proliferation in the gastric carcinoma cell line [30]. A derivative compound of coumarin, scopoletin, isolated from the *Macaranga gigantifolia* Merr. Leaves, show strong cytotoxic activities against cell lines of P388 murine leukemia [31].

A previous study reported that phenol has antiproliferative effects on several types of tumors. Phenol compounds of *Zingiber officinale* R. rhizome had shown cytotoxic activity in cancer cells through apoptosis [32]. The chemopreventive effects of phenol had been reported for having inhibition of proliferation effects, cell cycle blocking and apoptosis induction in different tumor cell lines [33,34]. One phenolic compound, named rosmarinic acid, found in Labiatae herbs, had a significant effect in inhibiting the cell proliferation in both the G0–G1 and G1–S phases induced by TNF-α and PDGF [35].

Triterpenoid compounds themselves have the property of inhibiting tumor growth. *C. gigantea*-derived triterpenoids could inhibit tumor growth by increasing cell apoptosis, as evidenced by the results of previous study [36]. A triterpenoid-rich extract of bamboo shavings and its main component, friedelin, had effective antitumor activities to inhibit the growth of P388 and A549 cancer cell lines [37].

Subfraction C_2 contained triterpenoid compounds, where similarities with taraxerol acetate compounds were found based on the FTIR and mass spectrometry spectral profiles. Taraxerol acetate is a triterpenyl ester compound with the molecular formula $C_{32}H_{52}O_2$, which has a molecular weight of 468.76 g/mol [21]. The chemical structure of taraxerol acetate has been presented in Figure 3a.

A study reported that steroid compounds may function as anti-inflammatory agents, and are useful in inhibiting prostate cancer [38]. A steroid fraction of *Brassica campestris* L. bee pollen chloroform extract displayed strong cytotoxicity in human prostate cancer PC-3 cells by triggering apoptosis [38]. Methanol extract of root bark of *C. gigantea* was reported to contain steroid group compounds, namely stigmasterol and β-sitosterol [39]. The stigmasterol antitumor activity towards Ehrlich Ascites Carcinoma (EAC) might be attributed to activation of the protein phosphatase 2A that causes apoptosis [40]. β-sitosterol has

shown a cytotoxic effect by interfering with multiple cell-signaling pathways, including cell cycle, apoptosis, proliferation, invasion, angiogenesis, metastasis and inflammation [41].

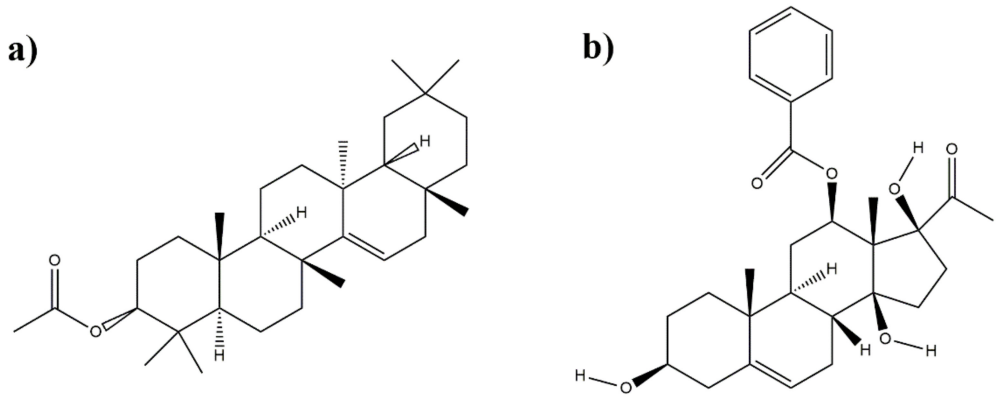

Figure 3. Structures of (**a**) taraxerol acetate and (**b**) calotropone.

Subfraction D_1 contained steroid compounds that produce yellowish-white solids and are characterized using FTIR and ^{1}H-NMR, showing similarities with the calotropone compound (Figure 3b) as found before, in which its structure was identified as 12β-O-benzoyl-3β,14β,17β-trihydroxypregnane-20-one and has 468.59 g/mol [14].

The results of the MTT Assay against P388 murine leukemia cell lines in this study showed that subfraction C_2 and D_1, respectively containing taraxerol acetate and calotropone, had IC_{50} of 50.64 and 49.33 µg/mL, respectively. Nonetheless, the ethyl acetate extract is the most potent, with IC_{50} as low as 21.79 µg/mL. Based on the National Cancer Institute (NCI) provisions, an extract is declared to be highly active as in anticancer if it has an IC_{50} value of <30 µg/mL and is moderately active if 30 µg/mL $\leq IC_{50} \leq$ 100 µg/mL [42]. Hence, both subfractions have a moderate anticancer activity. Meanwhile, the ethyl acetate is high. Higher antiproliferative activities in the ethyl acetate extract were ascribed to the synergism among the secondary metabolites [43].

It is worth mentioning that taraxerol acetate has been reported to have antitumor effects towards human glioblastoma cells [44]. Taraxerol acetate derived from methanolic extract from Hibiscus rosa sinensis leaves and stems is also effective against the K562 Leukemia cell line [45]. The steroidal aglycone (pregnanone), also called calotropone, isolated from ethanol extract from *C. gigantea* L. root, has shown an inhibitory effect on the cell lines of chronic myelogenous leukemia K562 and the human cancer SGC-7901 in the previous study [14].

5. Conclusions

Ethyl acetate extract from *C. gigantea* root bark contains steroids, terpenoids, coumarins, saponins and phenol compounds. Compounds from the aforementioned classes synergistically contribute to increase the antiproliferative activity against the P388 murine leukemia cell lines. The synergism is responsible for a higher antiproliferative activity of ethyl acetate than its isolates. We recommend further study into the ethyl acetate extract from *C. gigantea* root bark for the identification of its bioactive compound constituents.

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