Cytotoxic Activity of Ethyl Acetate Extract of *Calotropis gigantea* L. Stem Bark and its Fractions against P388 Cells

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Abstract. The present study deals with the cytotoxic activity of ethyl acetate extract of *Calotropis gigantea* L. stem bark and its fractions such as A, B, C, D and E fractions on murine leukemia cell line P388. Qualitative screening of ethyl acetate extract of stem bark of *Calotropis gigantea* L. for secondary metabolites showed the presence of phenolics, alkaloids, steroids, flavonoids, saponins and coumarins. Cytotoxic activity of the ethyl acetate extract of this plant and A, B, C, D and E fractions was conducted on P388 cells through MTT assay, with IC$_{50}$ value 57.05 μg/mL, 60.37 μg/mL, 55.65 μg/mL, and 58.26 μg/mL respectively, while D and E fractions less active. However the potential of the active fractions as indicated by the results in this study needs to be further investigated.

Keywords: *Calotropis gigantea*, secondary metabolites, cytotoxicity, P388 cells.

INTRODUCTION

Tumors and cancers are classified as diseases those are potentially dangerous to human life. The World Health Organization (WHO) reported deaths from the cancer by about 13% every year [1]. The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation [2]. According to the International Agency for Research on Cancer (IARC) in 2008 worldwide, it was estimated that there were 12.7 millions new cases, 7.6 million deaths; of these number, 56% of the cases and 64% of the deaths occured in the economically developing countries [3,4]. 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 [5]. 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*. It is a plant that originated in India and spread throughout tropical and subtropical regions of Africa and Asia. In ethnobotany leaves of *C. gigantea* believed to treat itching, trachoma, cough, constipation, and scabies; its flower is used as an asthma medication, nausea, and abdominal pain. While the latex is used to cure carbuncle, ekzema, toothache, syphilis, inflammation of the child’s ears, dysentery and swelling. 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. Especially regarding to its chemical content. Previous studies conducted by* 6 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, gigantsin 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 mg/mL and 6.7 mg/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.
Methanol extract of the roots of this plant are used as larvacidal for *Tribolium castaneum* [7]. This plant has the same family with the plant *Calotropis procera* (Asclepiadaceae) which has been studied that the latex at a dose of 1000 mg/mL can kill 100% third instar larvae of *Aedes aegypti* in 24 hours [8], which showed that the latex of this plant is very toxic. 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 [9]. Further research has been carried out fractionation of the ethyl acetate extract 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* 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. 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 bark of *C. gigantea* is taken as much as ± 2 kg. Furthermore, the bark is macerated with ethyl acetate solvent for 3x24 hours in order to obtain the extract solution. Maceration is repeated until the extracts obtained were almost clear. Then, the process followed by filtering and the filtrate was concentrated by using a vacuum rotary evaporator to produce concentrated ethyl acetate extract. Then, the weight of the extract is measured.

**Fractionation of Concentrated Ethyl Acetate Extract**

Concentrated ethyl acetate extract eluent system specified with a suitable solvent comparison using a TLC plate. Subsequently, the sample was drawn as much as 10 grams and its components are separated using a Vacuum Liquid Chromatography (VLC). Stationary phase in the form of silica gel, i.e. Silica gel 60 G which is as much as 100 grams and a mobile phase of dichloromethane and methanol 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 similarity of stain patterns after eluted with eluent system obtained and also sprayed with reagent seric sulfate. This is called the combined fractions. Then, the concentrated extracts and combined fractions are tested to identify chemical constituents and MTT assay to P388 cells.

**Qualitative phytochemical screening**

Chemical tests were performed on the ethyl acetate extract of stem bark of *C. gigantea* and its combined fractions using standard procedure to identify the phytoconstituens [10].

**MTT Assay**

Cytotoxicity assay was performed as the method that has been reported earlier [11,12]. 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 ethyl acetate extract from the stem bark of *C. gigantea* and combined fractions of ethyl acetate extract). After incubation for 72 h, 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 minutes. Absorbance reading is using microplate reader at $\lambda$540 nm and the fraction of surviving cells was calculated. Artonin E (100 mg) was used as a reference drug. Inhibition of the cell is calculated as follows:

$$\% \text{ Cytotoxicity} = (1 - \frac{\text{Abs test}}{\text{Abs control}}) \times 100$$
RESULT AND DISCUSSION

Maceration of the dried stem bark of *C. gigantea* in ethyl acetate yielded a brownish green extract. Fractionation of the extract by VLC on silica gel gave 14 fractions (FIGURE 1). From TLC analysis, according to similarity of stain patterns, the fractions were combined into five combinations which were fraction A (fraction 1-3), fraction B (fraction 4), fraction C (fraction 5-10), fraction D (fraction 11-12) and fraction E (fraction 13-14).

Phytochemical compounds were screened in the ethyl acetate extracts of stem bark of *C. gigantea* and its fractions through qualitative method. The results indicated the presence of alkaloids, saponins, phenols, steroids, flavonoids, coumarins and the absence of terpenoids in the extract. From the fraction A-E showed that the steroid, flavonoids, phenolic, and coumarins were concentrated in the fraction B, while the alkaloids, steroids, saponins and coumarins were found in the fraction C, the coumarins were concentrated in the fraction A. Then, the fraction D contains saponins and significant quantities of steroids, while the fraction E contains only the saponin which was shown in table 1.

**TABLE 1.** Phytochemical screening of ethyl acetate extract of stem bark of *C. gigantea* and its combined fractions

<table>
<thead>
<tr>
<th>No.</th>
<th>Secondary Metabolites</th>
<th>Ethyl Acetate Extract</th>
<th>Combined Fractions A</th>
<th>Combined Fractions B</th>
<th>Combined Fractions C</th>
<th>Combined Fractions D</th>
<th>Combined Fractions E</th>
<th>Observation Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Meyer’s reagent</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff’s reagent</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Red precipitate</td>
</tr>
<tr>
<td></td>
<td>Wagner’s reagent</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Brown precipitate</td>
</tr>
<tr>
<td>2.</td>
<td>Steroids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Green colour</td>
</tr>
<tr>
<td>3.</td>
<td>Terpenoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Saponins</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Honeycomb froth for a stable 30 minutes</td>
</tr>
<tr>
<td>5.</td>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Pale red /Purple</td>
</tr>
<tr>
<td>6.</td>
<td>Coumarins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Flouresenced</td>
</tr>
<tr>
<td>7.</td>
<td>Phenols</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Pale red</td>
</tr>
</tbody>
</table>

+: presence; - : absence

**TABLE (2).** MTT assay of ethyl acetate extract of *C. gigantean* stem bark and its combined fractions
The results from MTT assay indicated that the ethyl acetate extract and fraction A, B, and C have cytotoxic activity against murine leukemia P388 cells with value $IC_{50}$ as follow 57.05 µg/mL, 60.37 µg/mL, 55.65 µg/mL, and 58.26 µg/mL which was shown in Table 2, while fractions D and E less active. The Fraction B is most toxic to cancer cells in vitro compare to the other fractions, it might be phytochemicals analysis revealed the presence secondary metabolites has the most prominent amount in the fraction B. Phytochemical screening revealed the presence of various chemical constituents, which posses strong antioxidant activities. The antioxidant may prevent and cure cancer and other diseases by protecting the cells from damage caused by free radicals-the highly reactive oxygen compounds [13,14].

**CONCLUSION**

The phytochemical analysis revealed the bioactive metabolites which are responsible for the cytotoxicity in the ethyl acetate extract of *C. gigantea* stem bark and three fractions A, B and C against P388 cells. MTT assay results from the ethyl acetate extract and five combined fractions against P388 cancer cells is found that the ethyl acetate extract and three fractions combined (A, B and C) have cytotoxic activity with $IC_{50}$ value was 57.05 µg/mL, and fractions A, B , and C are as follows: 60.37, 55.65 and 58.26 µg/mL. Further study is required to isolate the lead compound responsible for this activity and to investigate cytotoxic activity to P388 cell lines for the development of new anticancer drug.

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