INTRODUCTION

Cancer disease has developed into a serious problem of health because of its increasing occurrences year by year. World Health Organization (WHO) stated that there are more than 10 million cancer cases every year in the world. In the United States, colorectal cancer is reported to be the third most common cancer in both men and women. There were 106,480 cases of colon and 40,340 cases of rectal cancer had been founded 2012. In 2009, there were 50,830 deaths from colorectal cancer. These mortality cases were totaled in almost nine percent of all cancer deaths [1]. Despite advances in the management failure, particularly through chemotherapy, can happen because of low selectivity of the cytotoxic and uncertainty degree of the molecular targeted. Several studies have been conducted to shed light on the effects and functions of the cytotoxic agent from the medicinal plant as a means to prevent and treat colon cancer.

Calotropis gigantea (Apocynaceae) is a medicinal plant that are widely grown in Asia, especially in Indonesia. Bangladesh, China, Burma, Malaysia, Pakistan, Philippines, Thailand and Sri Lanka. The plant has been traditionally used for treating inflammation, abscess, scabies, cough, trachoma, constipation, asthma, toothache, gastritis, otitis media, and dysentery [2]. Previous studies investigated several pharmacological properties of this plant. For instance, the leaves of this plant were reported to be effective for anti-diarrhea treatment [3], antibacterial remedy [4], antifungal [5], and antioxidant activity [6]. The flowers were shown to be useful for antibacterial and antifungal activity [7], the cytotoxic agent [8], and analgesic procedure [9]. Roots were claimed to be potent for antipyretic conditioning [10], cytotoxic agency [8], antimicrobial treatment [11], insecticidal activity [12], wound healing activity [13], CNS activity [14], and pregnancy interceptive properties [15]. The latex of the plant exhibited to contain purgative properties and procoagulant agencies [16], effective within wound healing processes [17], and antifungal remedy [18]. The stem was also reported to possess hepatoprotective effects [19].

In Previous study Calotropis gigantea roots extract (IC50=3.3, 7.0 mg/ml), Coroglaucigenin (IC50=4.7, 141 mg/ml) and Frugoside (IC50=34.65 mg/ml) showed significant activity against K562 and SG799101 cell lines [20]. Active compounds from roots that have been isolated are 19-dihydrocalactin (IC50=0.026 µM, 0.047 µM), calactin (IC50=0.022 µM, 0.028 µM), calotropin (IC50=0.029 µM, 0.046 µM) showed cytotoxic activity against human A549 and Hela cell lines [21]. In this study, we investigated the cytotoxic effects of fractions within the ethanolic extract of Calotropis gigantea leaves on colon cancer WiDr cell line.

MATERIALS AND METHODS

Materials

Calotropis gigantea leaves were collected and identified in Lembaga Ilmu Pengetahuan Indonesia (LIPPI) Purwodadi, East Java, Indonesia. Materials collected for cytotoxic assay were identified as [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical, St Louis, MO), H2O2 (Lab Vision Plus), chromogen 3, 3-diaminobenzidin (DAB) (Novo Castra). Preparations of ethanolic extract and fractions of Calotropis gigantea

Preparation of ethanolic extract and fractions of Calotropis gigantea

Dried powder of Calotropis gigantea was extracted using ethanol 70% with a ratio of 1:10 for 72 h. Then, the filtrate obtained was filtered while the sediment was re-extracted using ethanol 70% at a ratio of 1:8 for 72 h. The re-extraction was performed twice. The extract was then collected and evaporated under reduced pressure to give viscous ethanolic extract. The extract was added with 100 ml aqua dest and then mixed to yield a liquid form of ethanolic extract. The extract was fractionated with dichloromethane at a ratio of 1:1, resulting in the dichloromethane fraction and residue. The residue then fractionated using ethyl acetate at a ratio of 1:1 and yielded in ethyl acetate fraction and residue. The residue was then fractionated using butanol at ratio 1:1 with the result of butanol fraction and residue. The residue was categorised as water fraction.
Human colon cancer cell lines

Human colon cancer WiDr was kindly provided by Prof. Masashi Kawauchi, Nara Institute Science and Technology (NAIST). The cells were routinely grown in DMEM containing 10% FBS, 1% Penicillin-streptomycin (v/v), and L-glutamine (1 mmol) at 37 °C and 5% CO₂.

Cytotoxic assay

The human colon cancer WiDr cell lines were cultured in 96-well plates (Becton Dickinson Co., NJ, USA), and each well contained the 5x10³ cells. The culture cells were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO₂ and 95% air for 24 h. The cell confluence or the crowding of cells in the plate was about 70-80%. After a 24-hour incubation, the culture medium was discarded. The cells were treated by Calotropis gigantea extract and fractions (within treatment groups) or the vehicle (control group), and then incubated for 24 h. 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 h in 37 °C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, the stopper of 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 h in room temperature and protected from light. After incubation, the cells were shaken, and cells absorbance was measured using ELISA reader at λ595 nm.

Data analysis

The experimental data was the absorbance of each well, which was then converted to a percentage of the viable cells as described below.

Percentage of viable cells = [B-C/A-C] x 100%.

A, B and C were absorbance of control group, treatment group and medium, respectively. The potency of cytotoxic effect was represented by IC₅₀ value and calculated using probit analysis. IC₅₀ value represents a concentration of the fractions that produce cells’ death of 50%. Calculation of IC₅₀ values was based on a linear regression correlation between logarithms of concentration versus probit value of the percentage of cell viability.

Thin layer chromatography (TLC)

Detection and separation of active compound of crude extract and its fractions with TLC were carried out using silica gel F₂₅₄ in stationary phase and chloroform: methanol (95:5 v/v) in the mobile phase.

Statistical analysis

All data were classified as mean±SD. One method of variance analysis (ANOVA) followed by the least significant difference (LSD) test was used for statistical analysis.

RESULTS AND DISCUSSION

In this study, Calotropis gigantea leaves were extracted using ethanol 70% that can extract polar, semi-polar, and nonpolar compounds. Subsequently, the ethanolic extract was fractionated gradually with dichloromethane, ethyl acetate, and butanol and resulted in four fractions. These fractions were dichromomethane fraction, ethyl acetate fraction, butanol fraction, and a water fraction. The procedure to gain these fractions was described in fig. 1. This research sought to determine the impact of these fractions on WiDr cell line. The potency of cytotoxic effects of each fraction was then compared using IC₅₀ values.
Effect of ethanolic extract and fractions on WiDr colon cancer cell line

The potency test of ethanolic extract and fractions of *Calotropis gigantea* leaves was performed on WiDr cells as a model of human colon cancer. The concentrations of the extract used were 1, 10, 100, 500, and 1000 μg/ml. Cell viability was examined using MTT reagent after a 24 h incubation. Fig. 3 displays the effect of a series of concentration: ethanolic extract, dichloromethane fraction, and ethyl acetate fraction. These fractions were able to decrease the cell viability significantly (p<0.05) in a concentration-dependent manner. Butanol fraction and water fraction did not decrease the cell viability (p>0.05). IC50 value of ethanolic extract, dichloromethane fraction, ethyl acetate fraction, butanol fraction, and water fraction were 48.5 μg/ml, 40.57 μg/ml, 41.79 μg/ml, 737.74 μg/ml, and 8493 μg/ml respectively (table 1). The findings indicate that dichloromethane and ethyl acetate fraction were more potent than the other fractions.

The TLC profile on fig. 2 shows that ethanolic extract, dichloromethane and ethyl acetate fraction contained flavonoid and terpenoid compounds. Such compounds have been known to have an anticancer potency. Isorhamnetin-3-O-rutinoside, Isorhamnetin-3-O-Glucopyanoside, Tanaxasteryl acetate are a flavonoid compound of *C. gigantea* leaves [22]. The leaves of *C. gigantea* contain a cardenolide glycoside compound. The compound consists of 12α-hydroxy coroglucigenin, calotxin/calotropin, calotropigenin. Its compound demonstrated a cytotoxic potency against KB, MCF7 dan, NCI-H187 cell lines [23]. Calotropin from *Calotropis procera* have been known to have cytotoxic potency against Leukemia K562 Cell lines by the mechanism of cell cycle regulations with inhibited G2 phase and caspase-3 activation [24].

![Table 1: The IC50 value of cytotoxic of ethanolic extract and fractions of *Calotropis gigantea* leaves on human colon cancer WiDr cell](image)

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC50 [μg/ml]±SD</th>
</tr>
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<tbody>
<tr>
<td>Ethanol extract</td>
<td>48.50±2.39</td>
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<tr>
<td>Dichloromethane fraction</td>
<td>40.57±8.74</td>
</tr>
<tr>
<td>Ethyl acetate Fraction</td>
<td>41.79±8.48</td>
</tr>
<tr>
<td>Butanol Fraction</td>
<td>737.74±25.38</td>
</tr>
<tr>
<td>Water Fraction</td>
<td>8493±30.48</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>9.27±0.57</td>
</tr>
</tbody>
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Values are mean±SD of triplicates

![Fig. 3: Effect of ethanolic extract, dichloromethane fraction (DCM), ethyl acetate fraction (EA), butanol fraction (BuOH) and water fraction of *Calotropis gigantea* on WiDr cell viability. Cells were incubated for 24 h with various concentrations of ethanolic extract. Cell proliferation was examined using MTT assay](image)

![Fig. 4: The cell morphology of ethanolic extract at a dose of 10 μg/ml (A), Doxorubicin at a dose 50 μg/ml (B), dichloromethane fraction at dose of 10 μg/ml (C), ethyl acetate fraction at a dose of 10 μg/ml and (D), butanol fraction at a dose of 10 μg/ml (E), and water fraction at a dose of 10 μg/ml (F). The cellular morphology of cell was examined under a microscope (x400). The result was obtained from one representative data of nine experiments that demonstrated a similar result. Each point represented the mean±SD of nine experiments](image)
CONCLUSION
We concluded that ethanolic extract, dichloromethane fraction, and ethyl acetate fraction exhibited a potent cytotoxic on human colon cancer WiDr cell line. Thus, these extracts were potential to be developed as an anticancer agent in human colon cancer therapy.

ACKNOWLEDGEMENT
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CONFLICTS OF INTERESTS
Declared none

REFERENCES


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