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Research Article

CYTOTOXIC ACTIVITY EVALUATION OF *ERIOCAULON CINEREUM* R.BR. ON HELA AND VERO CELL LINES

PINUS JUMARYATNO[1]*, ARDE TOGA NUGRAHA[1], ADILIA TRI HIDAYATI[1], BAIQ RISKY WAHYU LISNASARI[1], WIDYANUR MAYA DIAHANDARI[1], NANANG FAKHRUDIN[2]

[1]Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia. [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. Email: pinus.jumaryatno@uii.ac.id

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ABSTRACT

Objective: This study aimed to evaluate the cytotoxic activity of the extracts and fractions of *Eriocaulon cinereum* against HeLa and Vero cell lines, which represent cervical cancer and normal cells, respectively. In addition, a phytochemical screening was carried out to determine the chemical constituents in the extracts and the active fractions.

Materials and Methods: The extracts of *E. cinereum* were obtained by ultrasound-assisted extraction method using *n*-hexane, ethyl acetate, and methanol, successively. The active extract was fractionated using vacuum liquid chromatography with dichloromethane followed by ethyl acetate. The cytotoxic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay method and was measured using microplate reader at the wavelength 595 nm. The data were analyzed with PROBIT from SPSS 16 for Windows®. In addition, phytochemical screening was performed using standard procedures.

Results: The cytotoxic evaluation of the extracts of *E. cinereum* showed that the ethyl acetate extract was the most active extract against HeLa cell line with the half maximal inhibitory concentration (IC_{50}) value of 580.07 µg/ml. The dichloromethane and ethyl acetate fractions from the active extract of *E. cinereum* exhibited cytotoxic activity against HeLa cell with the IC_{50} values of 466.61 µg/ml and 267.34 µg/ml, respectively. In addition, the ethyl acetate fraction showed a low cytotoxic effect against Vero cell line. The phytochemical screening of the ethyl acetate fraction indicated the presence of terpenoids and alkaloids.

Conclusion: This finding revealed the anticancer potential of *E. cinereum* and warranted further investigation for the discovery of new anticancer agents from natural resources for cervical cancer.

Keywords: Cytotoxic, *Eriocaulon cinereum*, HeLa cell line, Phytochemical screening, Vero cell line.

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INTRODUCTION

Cervical cancer is a cancer commonly caused by the human papilloma virus. The World Cancer Report 2014 reported that cervical cancer is the fourth most common cause of death in women affected by cancer [1]. While in Indonesia, according to the data from the Ministry of Health of the Republic of Indonesia, the prevalence of cervical cancer is 0.8% of the total population of Indonesia [2]. Therefore, cervical cancer is one of the priorities for the Indonesian Ministry of Health in terms of combating cancer.

Until now, the most common therapy for patients with cervical cancer is a surgery together with a radiotherapy for the early stage of cancer or a combination of radiotherapy and chemotherapy for advanced stage. The drugs commonly used for chemotherapy in the treatment of cervical cancer include cisplatin, carboplatin, paclitaxel, topotecan, and gemcitabine [3-7]. However, chemotherapy will cause unwanted side effects such as nausea, vomiting, hair loss, mouth sores, fatigue, and appetite loss [8].

Nature is known to have a rich source of natural compounds that have the health benefits to treat cancer, including the plant from *Eriocaulon* genus. Previous reports showed that some *Eriocaulon* species have the cytotoxic activity. For example, *Eriocaulon australe* from China has cytotoxic activity against A549 lung cancer cells, MCF-7 breast cancer cells, and HeLa cervical cancer cells [9]. Meanwhile, *Eriocaulon sieboldianum* was reported to exhibit cancer activity against HL-60 human leukemia cell line [10] and induce cell cycle arrest of human liver cancer cells HepG2 [11]. Furthermore, Fan *et al.* also reported antitumor activity against K562 leukemia cells from *E. sieboldianum* [12]. In addition, *E. sieboldianum* has been used as adjuvant therapy to treat cervical cancer in traditional Chinese medicine [12].

Nugraha *et al.* [13] conducted a preliminary study to screening the *Eriocaulon cinereum* R.Br. from Bangka Belitung Island, Indonesia, for anticancer. This plant has been used by local people to prevent and treat uncontrolled cells growth. The result showed the inhibition of HeLa cells growth with the half maximal inhibitory concentration (IC_{50}) value of 428 µg/mL on ethanol extract [13]. Based on this result, further investigation of *E. cinereum* was carried out as a source for new anticancer therapeutics. Therefore, the aims of this study were to investigate the cytotoxic activity of the extracts of *E. cinereum* and its fractions against HeLa cells and to identify the phytochemicals present in the active extract and fractions.

MATERIALS AND METHODS

Plant materials

The plant was collected from Parittiga, Jebus, Bangka Belitung Island Province, Indonesia, on July 2016. *E. cinereum* R.Br. was identified by Drs. Heri Sujadmiko and MSi. from the Laboratory of Plant Systematic at Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Voucher specimen was deposited at Laboratory of Pharmaceutical Biology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia (Collection No. BF-FAUII-01). Samples were dried using cabinet dryer at 50°C for 36 h and powdered.

Preparation of plant extracts

The extraction of plant material was done by ultrasound-assisted extraction method according to Mandal *et al.* [14] with some modifications. A total weight of 50 g powdered *E. cinereum* was macerated with 500 mL *n*-hexane using ultrasound-assisted extraction method at 40°C for 30 min. After filtering, the filtrate was collected and the residue was dried and then extracted 3 times with ethyl acetate (500 mL each) using the same method as in the extraction with *n*-hexane. After the third filtering process, the filtrate was again collected, and the residue was dried and macerated 3 times with methanol (500 mL each) in a similar fashion to the *n*-hexane maceration procedure. The filtrate obtained from each maceration process was concentrated using a rotary evaporator to give 0.05 g, 2.52 g, and 2.17 g of *n*-hexane, ethyl acetate, and methanol extracts, respectively.

Fractionation of ethyl acetate extract

The ethyl acetate extract (0.35 g) was fractionated using dichloromethane (750 mL) and ethyl acetate (600 mL) using vacuum liquid chromatography. The fractions obtained from the fractionation processes were evaporated to give dichloromethane (0.136 g) and ethyl acetate fractions (0.075 g).

Cell lines and cell cultures condition

HeLa (cervical adenocarcinoma) and Vero (African green monkey kidney) cell lines were provided by Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. The HeLa cell line was cultured in a complete medium consisted of Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.5% Fungizone (Gibco Amphotericin B), while the Vero cell line was cultured in a complete medium consisted of M199 supplemented with 10% FBS, 2% penicillin-streptomycin, and 0.5% Fungizone (Gibco Amphotericin B). Both the cells were incubated at 37°C in an incubator with 5% CO_2.

Cytotoxic evaluation

The extracts and fractions of *E. cinereum* R. Br. were evaluated with HeLa and Vero cell lines to evaluate the cytotoxic activity. HeLa and Vero cell lines (each cell with a density of 10^4 – 5×10^4 cells/mL) in 100 µL complete medium were seeded in 96 well plates. The cell cultures were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. After incubation, the complete media was discharged from the well and the cells were treated with *n*-hexane extract, ethyl acetate extract, and methanol extract of *E. cinereum* and dichloromethane and ethyl acetate fractions from ethyl acetate extract of *E. cinereum* with various concentrations (ranging from 15.625 to 1000 µg/mL). Prior to the treatment, the extracts and fractions of *E. cinereum* were dissolved in dimethyl sulfoxide. A series of concentration of 100 µL of extracts and 100 µL of fractions were applied to the cells then incubated at 37°C in 5% CO_2 incubator for 24 h. The cytotoxicity evaluation was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 100 µl of 0.5% MTT solution was added to each well of the treated suspension cells and then incubated for 4 h at 37°C in 5% CO_2 incubator. After 4 h, the 100 µL sodium dodecylsulphate (SDS) stopper (SDS 10% in 0.01 N HCl) was added to each well and further incubated at room temperature in a dark place for 24 h. The absorbance was measured by the enzyme-linked immunosorbent assay reader (Bio-Rad Benchmark Microplate Reader) at 595 nm. The cytotoxicity assay was performed in triplicate (n=3).

Phytochemical screening

The *n*-hexane, ethyl acetate, and methanol extracts of *E. cinereum* were subjected to preliminary phytochemical screening using the standard procedures analysis [15] to identify the various chemical constituents.

Qualitative chemical analysis was performed to screen the phytochemical contents in dichloromethane and ethyl acetate fractions from the ethyl acetate fraction of *E. cinereum* using thin-layer chromatography (TLC) and spray reagents. Fractions were spotted to TLC plate silica gel GF_{254} (Merck) and were eluted with *n*-hexane:ethyl acetate (3:2). The presence of compounds was detected by various spray reagents [16,17].

Statistical analysis

All the results of cytotoxic activity were analyzed with PROBIT from SPSS 16 for Windows®.

RESULTS

Cytotoxicity activity

To evaluate the cytotoxic activity of the extracts, the MTT assay was conducted and the IC_{50} values of the extracts were determined by measuring the concentration of the extract that caused 50% of cell population death. As shown at Table 1, the most active extract was the ethyl acetate extract with the IC_{50} value of 580.07 µg/mL, while the *n*-hexane extract showed no cytotoxic activity.

Due to the high cytotoxicity of the ethyl acetate extract, this extract was fractionated and evaluated for their cytotoxic activity against HeLa cell lines (Table 2). The dichloromethane and ethyl acetate fractions from ethyl acetate extract exhibited cytotoxic activity against HeLa cells with IC_{50} values of 466.61 µg/mL and 267.34 µg/mL, respectively. These fractions were also tested against Vero cells and showed IC_{50} values of 525.95 µg/mL and 795.59 µg/mL, respectively.

Phytochemical screening

The results of phytochemical screening of *n*-hexane, ethyl acetate, and methanol extracts of *E. cinereum* are displayed in Table 3.

Qualitative screening of the phytochemical content of the active fraction from ethyl acetate extract of *E. cinereum* was carried out with sprayed chemical reagents on the eluted sample with TLC. The results of the screening are presented in Table 4.

DISCUSSION

E. cinereum is a grass-like herbaceous plant growing in a watery swamp area [18]. This species can be found in Bangka Belitung Island, Indonesia, and traditionally, this herb is used by the local people for cancer prevention. *E. cinereum* has not been intensively studied for their property as anticancer compared to other genus of *Eriocaulon* [9-12]. The finding of the antiproliferative activity of the ethanol extract of *E. cinereum* in HeLa cells from our preliminary study [13] led to our further investigation to evaluate the cytotoxic activity of *E. cinereum* against HeLa cell lines.

In the present study, *E. cinereum* was successively extracted using *n*-hexane, ethyl acetate, and methanol to obtain the relatively nonpolar, semipolar, and polar extracts, respectively. The results of the cytotoxicity

Table 1: The cytotoxic activity of the extracts of *Eriocaulon cinereum* against HeLa and Vero cell lines

Extract	HeLa cell IC_{50} (µg/mL)	Vero cell IC_{50} (µg/mL)
n-hexane extract	>1000	>1000
Ethyl acetate extract	580.07±13.09	862.81±41.88
Methanol extract	626.41±14.29	758.21±14.77

IC_{50}: Half maximal inhibitory concentration

Table 2: The cytotoxic activity of the fractions from the ethyl acetate extract of *Eriocaulon cinereum* against HeLa and Vero cell lines

Fraction	HeLa cell IC_{50}(µg/mL)	Vero cell IC_{50}(µg/mL)
Dichloromethane	466.61±34.93	525.95±15.48
Ethyl acetate	267.34±4.19	795.59±8.98

IC_{50}: Half maximal inhibitory concentration

Table 3: Preliminary phytochemical screening of various extracts of *Eriocaulon cinereum* R.Br.

Phytoconstituents	Method	Extract		
		***n*-hexane**	**Ethyl acetate**	**Methanol**
Flavonoids	Shinoda test	+	+	+
	Zn+HCl test	−	−	+
Alkaloids	Dragendorff test	−	−	+
	Wagner's test	−	−	+
	Mayer's test	−	+	+
Saponins	Foam test	−	−	+
Terpenoids and Steroids	Liebermann's test	+	+	−

*Positive (+): Present, Negative (−): Absent

Table 4: Phytochemical Screening of ethyl acetate and dichloromethane fractions from ethyl acetate extract of *Eriocaulon cinereum* R.Br.

Phytoconstituents	Spray reagents	Fractions	
		Dichloromethane	**Ethyl acetate**
Flavonoids	$AlCl_3$	+	−
Alkaloids	Dragendorff	−	+
Terpenoids	Anisaldehyde H_2SO_4	+	+
Steroids	Liebermann–Burchard	+	−

*Positive (+): Present, Negative (−): Absent

evaluation of the extracts against HeLa cell showed that the ethyl acetate extract was the most cytotoxic with IC_{50} value of 580.07 µg/ml (Table 1). The phytochemical screening showed that the ethyl acetate extract of *E. cinereum* contains flavonoids, alkaloids, terpenoids, and steroids (Table 3). Previous chemical analysis reported that *E. cinereum* contains abundant of flavones and isoflavones with major characteristic compounds are hispidulin, iristectorigenin A, and irigenin [19]. In addition, hispidulin isolated from *E. australe* demonstrated cytotoxicity against Hela cells with IC_{50} value of 10.38±1.13 µg/ml [9].

The presence of flavonoids in our ethyl acetate extract led the assumption that this chemical constituent might contribute in the cytotoxic activity against HeLa cells. Therefore, a further investigation has been undertaken by fractionated the ethyl acetate extract of *E. cinereum* into dichloromethane and ethyl acetate fraction using vacuum liquid chromatography. The results showed that the cytotoxic activity of the fractions was higher than the ethyl acetate extract (Table 2) with the IC_{50} values of 466.61 µg/ml and 267.34 µg/ml for dichloromethane and ethyl acetate fractions, respectively. Interestingly, based on phytochemical screening (Table 4), flavonoids, terpenoids, and steroids were detected in the dichloromethane fraction, while terpenoids and alkaloids were present in the ethyl acetate fraction. This indicated that the cytotoxicity of *E. cinereum* against HeLa cell might be due to the presence of terpenoids and alkaloids instead of flavonoids. In addition, the lower cytotoxic activity of dichloromethane fraction might be due to the antagonistic effect possessed by the presence of other chemical constituents in the fraction which could prevent the activity of the flavonoid. Many compounds belonging to alkaloids [20-22] and terpenoids [23-25] from different plants have been reported for their cytotoxic activity against HeLa cell lines. However, to the best of our knowledge, the cytotoxic activity of terpenoids and alkaloids from *E. cinereum* against HeLa cells have not been reported yet.

The cytotoxity of the ethyl acetate extract of *E. cinereum* and its fractions to Vero cell lines were also evaluated. The results showed that the ethyl acetate extract, dichloromethane fraction, and ethyl acetate fraction have a weaker cytotoxicity against Vero cells with IC_{50} values of 862.81 µg/ml, 525.95 µg/ml and 795.95 µg/ml, respectively. This study indicated that the ethyl acetate fraction might contain compounds with a potential cytotoxic activity with a good selectivity and can be further developed as anticancer agent. Therefore, a further study is needed to identify the secondary metabolites that responsible for the activity and the mechanisms of extracts and pure compounds.

CONCLUSION

This study revealed that *E. cinereum* has the potential to be developed as anticancer agent for cervical cervix. The ethyl acetate fraction obtained from the ethyl acetate extract of the plant exhibited the most potent cytotoxic activity against HeLa cancer cells with low cytotoxicity against a normal cell, Vero.

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CONFLICTS OF INTERESTS

All authors have none to declare.

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