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Original Research Article

Anticancer Activity of Subfractions from *Eriocaulon cinereum* R.Br Extract in Cervical Cancer Cells

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ABSTRACT

The previous studies on *Eriocaulon cinereum* R.Br showed that one of the subfractions from its dichloromethane extract has better activity than its crude extract in a breast cancer cell line. Meanwhile, the activity of the same subfractions has never been studied in cervical cell lines. The hypothesis was that the subfraction activity was also better than its crude extract in cervical cancer lines. This study compared the activity of extracts, fractions, and subfractions of *E. cinereum* R.Br extracts on HeLa cervical cell lines. The sample extraction was carried out using ethyl acetate solvent and continued via fractionation using semi-preparative high-performance liquid chromatography (HPLC). The samples were then tested for cytotoxicity on HeLa cervical cancer cells and Vero cells as normal control cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Finally, thin-layer chromatography (TLC) was used to identify the active compound from the active subfractions. The data obtained from the cytotoxic activity evaluation were analyzed via linear regression using Excel for MS Windows®. The results showed that the best cytotoxicity against HeLa cells was with the ASA012 subfractions with an IC_{50} value of 83.248 µg/mL and a selectivity index (SI) of 2.157. These compounds were obtained from a peak with a retention time of 19.605 min and identified as terpenoids. These subfractions have moderate cytotoxicity activity and have better activity than their crude extract. Therefore, it is necessary to carry out further isolation to separate them to obtain pure compounds with better cytotoxic activity.

Keywords: *Eriocaulon cinereum* R.Br, HeLa Cells, MTT Assay, Cytotoxic Compounds

Introduction

Cancer is one of the leading causes of death worldwide, with an estimated 1 in 6 deaths, according to the World Health Organization. Cervical cancer is one type of cancer that has a high incidence in women worldwide.[1] In 2018, cervical cancer affected 570,000 people, with 311,000 deaths.[2,3] Indonesia also has a high incidence of cervical cancer, with a disease occurrence of 9.2% of the population and a mortality rate of 9%.[4] The incidence and mortality rate, which is increasing year to year and is even predicted to increase to 54.9% in the next 20 years, is thought to be due to the ineffectiveness of treatment of cancer patients.[5–7] Moreover, most currently available therapies cause unpleasant effects for patients, thus reducing patient compliance during treatment.[8–11] Therefore, developing new therapies that are more efficient and have selectivity in inhibiting the growth of cancer cells without damaging healthy tissues is essential.

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Natural compounds are one source that can be used to obtain alternative treatments and even new medicines to overcome cancer.[12,13] The *Eriocaulaceae* family is known as a source that has activity against cancer. As part of this family, *Eriocaulon sieboldianum* and *Eriocaulon australe* are used as adjunctive therapy for cancer in China. Both can inhibit cell proliferation and induce apoptosis.[14,15] Another species called *E. cinereum* has also been used as a traditional medicine to treat cancer by people in the Bangka Belitung area. This species, widely found in America, Africa, Australia, and Asia, can grow in muddy locations, has membranaceous leaves, trimerous flowers, and staminate flowers with completely fused sepals, and has been reported to be weeds in rice fields.[16–19]

Previous research showed that the purer the content of *E. cinereum* is, the better the toxicity on MCF-7 and T47D breast cancer cells. In a cytotoxic test on MCF-7, ethyl acetate extract, the extract with the best activity after purification by fractionation using ethyl acetate, showed better cytotoxicity with an IC_{50} value of 214.75 µg/mL. The active isolate that was successfully isolated from the fraction, which is a flavonoid isolate, has an IC_{50} of 7.28 µg/mL.[20–22] The same results were also found upon testing toxicity on T47D cells. The subfraction containing terpenoids had a lower IC_{50} compared to the dichloromethane fraction, with IC_{50} values of 84.8 and 131.921 µg/mL, respectively.[23,24]

However, research using this species on HeLa cervical cancer cells has only been done at the fraction level, with the best IC_{50} being in the ethyl acetate fraction with an IC_{50} of 235.65 µg/mL.[25,26] Based on previous research, we hypothesized that the subfraction and isolates from this fraction would have better activity than the crude extract on HeLa

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cervical cancer cells. Therefore, in this study, we compared the cytotoxic activity of *E. Cinereum* fractions, subfractions, and isolates on HeLa cervical cancer cells and calculated their selectivity on normal Vero cells, followed by the identification of active compounds in the active isolate.

Materials and Methods

Plant collection

The *E. cinereum* sample was collected from Bangka Belitung Province in March 2020 and was determined at the Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta. The voucher specimen was deposited at the Laboratory of Pharmaceutical Biology, Department of Pharmacy, University Islam Indonesia, Yogyakarta, Indonesia (No. BF-FAUII-01).

Plant extraction and identification

E. cinereum was dried in a cabinet dryer at 50°C for 36 h, and the sample was prepared as a powder. Then, extraction was performed by ultrasound-assisted extraction according to Mandal *et al*. (2015) [27], who used 200 g of powder and extracted alternately to produce n-hexane, ethyl acetate, and methanol extracts.[27] The ethyl acetate extract was then fractionated using the vacuum liquid chromatography method with dichloromethane and ethyl acetate as solvents. Further separation was then performed on ethyl acetate fractions (which has the best activity according to the previous study) using semi-preparative high-performance liquid chromatography (HPLC) (Waters®). The chromatography system used was a C18 column (Xterra®) as the stationary phase, water: acetonitrile gradient as the mobile phase, and PDA as the detector. Identification of the terpene compounds from these subfractions was then performed by thin-layer chromatography (TLC) and anisaldehyde-sulfuric acid reagent.

Cell lines and cytotoxic activity evaluation

HeLa and Vero cell lines obtained from the Cell Culture Laboratory Universitas Muhammadiyah Yogyakarta were cultured at the Cell Culture Laboratory, Department of Pharmacy, University Islam Indonesia. Cytotoxicity was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. All plates that were treated and incubated for 24 h were observed using an inverted microscope. Then, the stock solution for MTT reagent was prepared by dissolving 50 mg of MTT powder into phosphate buffer saline (PBS) to achieve a volume of 10 mL. The MTT reagent stock must be diluted first with complete media to obtain a 0.5 mg/mL concentration. The MTT reagent that had been diluted with media was placed into all wells, including cell control wells, positive control wells (doxorubicin), and media control wells, and then incubated in a CO_2 incubator at 37°C for 4 h. After the incubation process, cell conditions were observed using an inverted microscope to determine if formazan salt crystals had formed. The plate that had formed crystals of formazan salt was then added to a 100 L of 10% sodium dodecyl sulfate (SDS) as a stopper in 0.01 N HCl and incubated for 24 h in a dark location. After 24 h of incubation, the absorbance plate was read using a microplate reader (ELISA) at a wavelength of 570 nm. This study also used doxorubicin as the positive control.

Statistical analysis

Absorbance values from the MTT assay were used to calculate the percentage of viable cells. This value was then used to determine the linear equation from the graph using the % viability cells as y-axis and concentration on the x-axis using Microsoft Excel®. The IC_{50} value as the cytotoxicity parameter was then obtained from this equation.

Results and Discussion

After the fraction and subfraction step, four samples were used in this study. Two included the ethyl acetate fraction (ASA001) and dichloromethane fraction (ASA002) obtained from ethyl acetate extract, and the remaining included terpenoid subfraction/isolate (ASA011) and flavonoid subfraction/isolate (ASA012) obtained from the subfraction from the two previous fractions. The percentage yield for each samples was different (Table 1), and the subfraction/isolate ASA011 and ASA012 had a smaller percentage yield than the fraction (ASA001 and ASA002). Data from fractionation and sub-fractionation results showed more than 10% yield values for ASA001, ASA002, and ASA011. This confirmed the results of previous research, which showed that the ultrasound-assisted extraction method could be used as an alternative for extracting natural materials.[28,29] Moreover, the percentage yield for the fraction was much higher than for the subfraction, which meant that the subfraction contained fewer compounds or was purer than the fraction.

Recent research on a cytotoxic compound primarily related to cancer cells has still used the MTT method.[30,31] The results are shown in Table 2, and the data show that isolate ASA011, with a retention time of 19.605, has the best activity with an IC_{50} value of 83.248 g/mL against HeLa cells and a selectivity index of 2.157 against Vero cells. The results of cytotoxicity testing of the four samples showed that the ASA011 subfraction was the most active subfraction with an IC_{50} value below 100 µg/mL, classified as moderate cytotoxicity. These results are not excellent against cancer cells, but they still have potential as a treatment alternative for cancer patients. Moreover, if further purification is carried out, it is possible to obtain compounds with cytotoxic activities that are much better than ASA011. This possibility was obtained from the cytotoxicity results of the subfraction, which were much better than those for the fraction and extract. Thus, when the compound is purer, the cytotoxic activity is likely better. These results follow previous studies of MCF-7 or T47D cells, in line with the hypothesis proposed in this study.[20–26]

Table 1: Yield (%) values for the fraction and subfraction/isolate obtained in this study

Compound	Yield (%)
Ethyl acetate fraction (ASA001)	38.64
Dichloromethane fraction (ASA002)	29.91
Terpenoid subfraction/isolate (ASA011)	10.44
Flavonoid subfraction/isolate (ASA012)	5.95

Table 2: Cytotoxic activity (IC_{50}) and selectivity index (SI) values for the *E. cinereum* fractions and subfractions against MCF-7 and Vero cell lines

Compound	HeLa (IC_{50}; µg/mL)	Vero (IC_{50}; µg/mL)	Selectivity Index (SI)
ASA001	215.435	308.627	0.001
ASA002	163.670	413.042	2.524
ASA011	83.248	179.537	2.157
ASA012	132.796	213.457	1.607
Doxorubicin	11.137	66.994	5.988

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The compound selectivity index, which was also determined in this study, showed that the selectivity of compounds in normal Vero cells was 2.157, which is classified as being in a good category with a value >2. This shows that the compound is selective and can differentiate between cancer cells and normal cells. Thus, it only works on cancer cells and does not interfere with or kill normal cells. This good selectivity can be one solution to the side effects caused by anticancer compounds. These cause discomfort in patients and even reduce compliance with drug use, which can reduce the success of therapy.

The phytochemical test results showed that two peaks showed the best cytotoxic activity against HeLa cells. The first peak was at a retention time of 19.605, and the second was at a retention time of 32.502 (Figure 2). The maximum wavelength of the compound (Retention time (TR) of 19.605 at 219 nm) was determined using a photodiode array (PDA) detector. The group of compounds was determined from identification with spray reagents using anisaldehyde: sulfuric acid (Figure 1), which appears as a violet color, indicating that it contains a terpene compound. Purification of ASA011 compounds was carried out using preparative RP-HPLC (Reversed Phase-HPLC). Compound ASA011, found at a retention time of 19,605, has the best anticancer activity against HeLa cells. These compounds were identified using TLC and are known to be terpenoid compounds because of the violet color that appears after spraying with an anisaldehyde-sulfuric acid and then heating 100ºC. Moreover, the PDA detector on the RP-HPLC provides information on the maximum wavelength at 219 nm. We can conclude that terpenoid compounds from this study were the active cytotoxic agents against HeLa cells. These results align with previous research on T47D cells, which showed that the subfraction containing terpenoids had the best activity.[23,24] However, these results do not align with previous studies on MCF-7 cells, which showed that the subfraction with the best activity contained flavonoids.[20–22]

This discovery shows that more than one type of active compound, both subfractions of flavonoids and terpenoids, is contained in *E. cinereum* and can be developed as an anticancer compound. Flavonoid compounds as anticancer agents are also found in many other species of the same genus, namely *E. australe* and *E. sieboldianum*.[14,15] This compound is reported to inhibit the growth of cancer cells through the apoptotic pathway, which involves important proteins, such as p53, Bcl-2, Bax, caspase-3, and PARP.[15] The mechanism of terpenoids found in the *Eriocaulon* genus has not been previously reported. Thus, further research is needed to determine the structure of terpenoids, especially those contained in *E. cinereum*, and their specific mechanisms for inhibiting the growth of cancer cells, particularly cervical cancer cells.

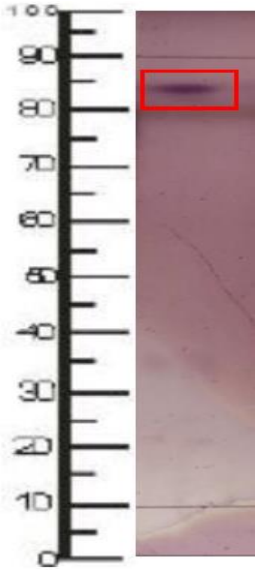

Figure 1: Compound identification using TLC. The TLC system used was as follows: mobile phase using n-hexane/ethyl acetate (8:2) and stationary phase using TLC plate GF60 254.

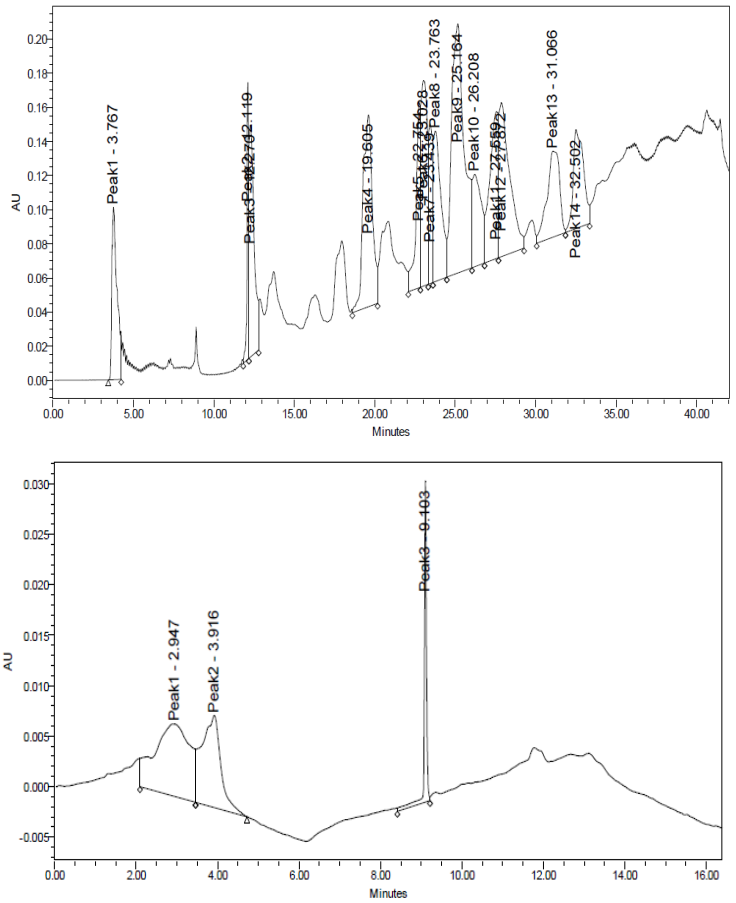

Figure 2: Chromatogram RP-HPLC with a photodiode array detector

Chromatogram of the fraction with active compounds TR 19.605 and TR 32.502 with a gradient mobile phase of 100% water to 100% acetonitrile (1 ml/ minute)

Identification of purity of the terpene at TR 9.103 with an isocratic mobile phase of 40% water: 60% acetonitrile (1 mL/min)

Conclusion

The ASA011 subfraction exhibited the best activity against HeLa cervical cells with an IC_{50} of 83.248 g/mL and a selectivity of 2.157. This shows that this subfraction can be developed as an alternative compound to fight cancer cells because it is classified as moderately cytotoxic. Further purification is needed for the terpenoid compounds contained in the ASA011 subfraction to obtain more active compounds against cervical cancer cells. Moreover, determining the structure of terpenoids and their anticancer mechanisms should also be carried out to develop these compounds as anticancer agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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