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Bioassay-guided Fractionation, LC/MS Analysis and *In Vitro* Cytotoxic Activity of *Eriocaulon cinereum* R. Br on T47D Breast Cancer Cells

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In this study, we examined the cytotoxic activity of *Eriocaulon cinereum* R.Br. against breast cancer T47D cells and normal Vero cells. The plant material of *E. cinereum* was extracted with *n*-hexane followed by a series of extraction with ethyl acetate and finally with methanol. The ethyl acetate extract was then fractionated with dichloromethane and ethyl acetate. The dichloromethane fraction was concentrated, then fractionate using a preparative TLC. The subfractions obtained probably contained terpenoids (SF1), flavonoids (SF2), and steroids (SF3) as the three major compounds based on staining reagent and MS identifications. Later, the subfractions; SF1–SF3 were subjected to cytotoxic activity on breast cancer T47D cells. The result showed that the IC_{50} values against T47D cells are 84.8 ± 7.8, 89.7 ± 3.4, and 135 ± 12 µg/mL, respectively. In contrast, IC_{50} values against Vero cells were > 300 µg/mL for all compounds. Among them, the SF1 subfraction shown the good cytotoxic activity compared to other subfractions and has the potential to be further developed.

Key words: *Eriocaulon cinereum* R. Br.; Rumput Gong; T47D cells; Cytotoxicity

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Cancer is the second leading cause of death globally. The most common types of cancer in men involve the lung, prostate, colon, and liver. Among women, breast, colorectal, lung, cervical, and thyroid cancer are the most frequent. Breast cancer is one of the cancer types exhibiting an increase in the mortality rate. It is the most diagnosed cancer in women, with 627,000 deaths worldwide in 2018 [1]. This rising incidence is not only observed in developed countries, but also in developing countries such as Indonesia where breast cancer is the most prevalent cancer and was responsible for 11% of total cancer deaths in 2018 [2]. The increased morbidity and mortality rates of breast cancer have spurred the development of innovative therapies with the aim of increasing survival and improving the prognosis of breast cancer patients. To date, the main therapies recommended for breast cancer patients are chemo- and radiotherapy. However, these therapies can kill both breast cancer and normal cells, resulting in decreased patient health and productivity [3]. In addition, in terms of pharmacoeconomic, breast cancer is classified as a life-threatening disease that involves large medical expenses [4].

Therapies applied in the treatment of breast cancer are often accompanied by side effects that can damage surrounding normal cells [5]. This is one of the main reasons for the need to find effective alternatives to current anticancer agents with minimal potential side effects. Furthermore, feasible and affordable therapy is an important factor when developing new anticancer treatments [1]. Various natural products have been used as alternative therapies in cancer to help the healing process and control the growth of cancer cells with minimal side effects [6]. Several metabolites from plants are known to have inhibitory effects on cancer cell activities, such as inhibiting cancer cell proliferation and inducing apoptosis [7]. As an example, people in China have used plants from the *Eriocaulon* genus as a therapy to prevent abnormal cell growth and as an adjuvant in tumor therapy [8]. In Indonesia, particularly in Bangka Belitung Islands, *Eriocaulon cinereum* R. Br. (locally known as rumput gong) has been used as an alternative therapy and it is believed to be effective against cancer. Thus, *Eriocaulon* genus plants are a potential natural resource to be investigated as a source of anticancer agents. This genus includes 435 species of plants that live in water or swamps around the globe. They have been reported to have the potential for treating cancer [9]. Previous study on *Eriocaulon sieboldianum* against the K562 myeloid leukemia cell lines, reported that it inhibited proliferation and

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induced p53 to regulate the apoptotic pathway [10]. In addition, Xu *et al*. (2013) found that flavonoid compounds isolated from *Eriocaulon australe* have cytotoxic activity against breast, cervical, and lung cancer cells [11]. A previous study showed that flavonoid compounds such as (2S)-3′,4′-methylenedioxy-5,-methoxy-7-flavan and hispidulin 7-O-β-D-(6-O-cinnamoyl)glucopyranoside were isolated from *Eriocaulon australe* [11]. Another study mentioned that several species of the genus *Eriocaulon* contained quercetagetin and gossypetin [12].

In Indonesia, *Eriocaulon cinereum* (*E. cinereum*), which lives and thrives on wet soils in Bangka Belitung Province, possesses cytotoxic activity against HeLa cells with an IC_{50} value of 427 μg/mL [13]. However, studies on the cytotoxic activities of these compounds remain limited, particularly against several cancer cell lines. Therefore, this research was designed to examine the cytotoxic activity of *E. cinereum* R. Br extracts against the T47D breast cancer cell line and to investigate the selectivity by analyzing its toxicity to the normal Vero cell line. Thus, it can be a promising anticancer based on the natural product that actively kills cancer cells with less adverse reactions.

EXPERIMENTAL

General Method

n-hexane, ethyl acetate, methanol and dichloromethane with technical grade obtained from JT Baker® and were utilized for extraction and fractionation. Thin-layer chromatography (TLC) was carried out using a pre-coated 4×10 cm aluminium plate, 0.25 mm thickness of silica gel 60 F_{254} (Merck, Germany) which will be visualized under UV-Vis's light (254 and 365 nm). Silica gel in vacuum liquid chromatography (VLC) was prepared using silica gel 60 of 230-400 mesh (Merck, Germany) depending on the weight of the extract or fractions. The LC-MS analysis was performed using Shimadzu LCMS 8030 Triplequadrupole ESI.

For cytotoxic evaluation, the reagent used were RPMI medium (Gibco®), Phosphate Buffer Saline (Sigma®), Penicillin-streptomycin 1.5% (Gibco®), doxorubicin (Sigma®), FBS 15% (Sigma®), MTT 0.5% (Bio Basic®), SDS stopper 10% (Merck®), trypsin-EDTA 0.025% (Gibco®), fungizone 0.25% (Sigma®), trypan-blue 1% (St. Cruz®), and DMSO (Merck®). While for formazan formation that correspond to the cell viability was determined with Elisa reader (Benchmark®).

Plant Material

E. cinereum was collected from Parittiga, Jebus, Bangka Belitung Islands Province, Indonesia, in January 2018. The plant was identified at the Laboratory of Plant Systematics at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. It was deposited as a voucher specimen (Code: BF-FAUII-01) at the Laboratory of Pharmaceutical Biology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia. The samples were sorted and dried at 50°C for 36 hrs, then ground into a powder.

Preparation of Samples of Plant Material

Extraction was done by using the modified ultrasound-assisted method [14]. A total of 50 g of dry material of *E. cinereum* was extracted with 500 mL *n*-hexane. The filtrate was collected, and the residue was extracted with ethyl acetate (500 mL). The procedure was repeated with methanol (500 mL), and each extraction process was carried out three times. All filtrates were concentrated using a rotary evaporator to obtain *n*-hexane, ethyl acetate, and methanol extracts. The ethyl acetate extract was fractionated using vacuum liquid chromatography (VLC) with dichloromethane (750 mL) and ethyl acetate (600 mL) as solvents. The dichloromethane and ethyl acetate fractions were then concentrated in a rotary evaporator. The dichloromethane fraction was purified using preparative thin-layer chromatography (PTLC) with *n*-hexane: ethyl acetate (8:2) as the mobile phase. Based on this, we obtained three subfractions (SF1– SF3) containing terpenoids (SF1), flavonoids (SF2), and steroids (SF3) based on staining reagent indicator.

Phytochemical Identification

Phytochemical identification was carried out on all extracts, fractions, and subfractions. The active fraction which is the dichloromethane extracts was proceed and identified by LC-MS (Shimadzu LCMS 8030 Triplequadrupole ESI) and compared with the library.

Cytotoxic Evaluation (MTT Assay)

Cytotoxic activity of the *E. cinereum* R. Br extracts against the T47D and Vero cell lines was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were suspended in the growth medium (Roswell Park Memorial Institute 1640), then centrifuged at 252 g for 10 min. The supernatant was removed, and the cells resuspended in 10 m medium followed by incubation in a CO_2 incubator at 37°C. Cells were observed under a microscope and harvested when they became confluent. Cells (10,000–50,000 cells/mL) were then seeded at 100 μL into 96-well plates. After 24 hrs, 100 μL of a dried extract dissolved in dimethyl sulfoxide at various concentrations up to 500 μg/mL were added and the plate was incubated for 24 hrs in a CO_2 incubator at 37°C. The medium was then removed from the well and 100 μL of fresh medium containing 10 μL MTT (0.5%) were added. The plate was incubated for 3 hrs and then 10% sodium dodecyl sulphate was added. The plate was incubated for a further 4 hrs in the dark at a room temperature before measuring the absorbance at 550 nm using a microplate reader at 550 nm. In addition, doxorubicin

was utilized as standard control. The percentage of cell viability was calculated according to equation 1.

$$\text{Viable cell (\%)} = \frac{(\text{Absorbance sample} - \text{absorbance blank})}{(\text{Absorbance control} - \text{absorbance blank})} \times 100 \quad \text{Eqn. 1}$$

Statistical Analysis

The percentage of cell viability was analysed using PROBIT from SPSS 16 for Windows® to determine the IC_{50} value. Data were expressed as means ± standard deviation. The comparison of IC_{50} value between T47D breast cancer and Vero cell resulted on selectivity index (SI).

RESULTS AND DISCUSSION

Bioassay-guided Fractionation and Phytochemical Analyses

The extracts, fractions, and subfractions was evaluated for its cytotoxic activity against T47D cell lines. A systematic bioassay-guided fractionation was carried out to identify compounds with potent cytotoxic activity. Among them dichloromethane fraction exhibits the good activity and able to suppress the growth of T47D cell lines in dose-dependent manner. Further fractionation of dichloromethane fraction using preparative thin-layer chromatography (PTLC) afforded three subfractions SF1–SF3. The qualitative phytochemical screening of the SF1–SF3 subfractions are shown in Table 1. The primary composition in these subfractions were terpenes, flavonoids, and steroids, respectively.

Furthermore, the dicloromethane fraction was injected into LC-MS system to give a chromatogram recorded at 223 nm. The separation of these compounds resulted 10 dominant compounds as shown at Table 2. These compounds were grouped based on the percentage of the number of compounds in the fraction namely polyketides (oleic acid), terpenoids (sabinene, *trans*-caryophyllene, dihydrocarvone, α-terpinolene, *trans*-β-farnesene, and isotanshinone II), and flavonoids ((*R*)-semivioxanthin). The fragmentation pattern of isotanshinone II and (*R*)-semivioxanthine were described in Figures 1 and 2, respectively.

Table 1. Phytochemical screening of *E. cinereum* subfractions

No.	Subfraction*	Phytochemical content	Description of observation
1	SF1	Terpene	The results of qualitative phytochemical screening with anisaldehyde: sulfuric acid showed that the sample contained terpenes at 0.35 R_f.
2	SF2	Flavonoid	The results of qualitative phytochemical screening with $AlCl_3$ showed that the sample contained flavonoid compounds at 0.60 R_f.
3	SF3	Steroid	The results of qualitative phytochemical screening with the Lieberman-Burchard reagent showed that the sample contained steroid compounds at 0.50 R_f.

*The mobile phase was n-hexane: ethyl acetate (8:2); the stationary phase was TLC Plate GF60 254.

Table 2. Identification of 10 major compounds in the dichloromethane fraction based on the Shimadzu LC-MS library

No.	t_R (Minutes)	Area	Area (%)	High	Compound
1	1.80	12389549	3.094	882041	Sabinene
2	4.39	14588135	3.643	408075	Isotanshinone II
3	4.79	14518374	3.625	401488	*trans*-Caryophyllene
4	5.54	12591608	3.144	385637	*trans*-β-Farnesene
5	6.66	11766044	2.938	366151	Dihydrocarvone
6	7.21	22043930	5.505	360126	Oleic acid
7	8.26	16684723	4.166	340340	(*R*)-Semivioxanthin
8	9.55	21409813	5.346	318952	(*R*)-Semivioxanthin
9	15.48	20533086	5.127	254339	(*R*)-Semivioxanthin
10	63.84	13213105	3.299	7150573	α-terpinolene

Since the subfractions contained terpenoids, flavonoids, and steroid, there was some correlation between anticancer activity and phytochemical compound content. This finding supports previous results using HeLa cells [13]. Flavonoid compounds, including flavanols, anthocyanidins, flavones, isoflavonoids, and apigenin have cytotoxic activity against many types of cancer cells [15, 16]. Their cytotoxic activity appears via activation of the caspase-3 pathway, prompting apoptosis [15]. Furthermore, an animal model revealed that the mechanism involved reactive oxygen species- and endoplasmic reticulum stress-dependent pathways that induced apoptosis [17]. The cytotoxic activity of apigenin (a flavone) has also been shown to involve free radical scavenging and promoting metal chelation in an *in vivo* tumor model [18]. In addition, it increased the glutathione concentration and enhanced endogenous defenses against oxidative stress [15].

Other active plant constituents are terpenoids, a class of secondary metabolites that is divided into monoterpenoids, sesquiterpenoids, diterpenoids, sesterpenoids, triterpenoids, and tetraterpenoids. The mode of action of those compounds, especially monoterpenoids, is by reducing the number of Ras-related proteins, G1 arrest, and the induction of apoptosis [19]. Steroids can also play an important anticancer role due to their ability to interact with various biological pathways. Most anticancer steroids are enzyme inhibitors, such as aromatase and sulfatase inhibitors for breast cancer [20]. In addition, several modifications on steroidal molecules can result in important anticancer lead compounds [21]. However, in the current study, the SF3 subfraction, which contained steroid compounds, displayed weak cytotoxic activity, possibly due to the short time frame of exposure in an *in vitro* system.

Analysis result on the MS spectrum at t_R 4.39 minutes (entry 2) showed an interesting pattern of compound fragmentation. In the positive ionization pattern, positive ion molecules were detected with the ionization character of isotanshinone II compound, which was observed in the form of fragmentation of the compound $[M+H]^+$. Additionally, fragments with the addition of CO_2 and H_2O groups were also examined. Giving the example, the protonated molecule m/z 278 $[M+2H]^+$ was determined with a 100% abundance percent in the MS spectrum as well as m/z 371 $[M+CO_2+3H_2O]^+$. While in the negative ionization pattern, molecular ions were characterized with the losing CH_3 and H groups. As in the m/z 243 molecule, its fragmentation lost 2 CH_3 groups and 3 H atoms $[M-2CH_3-3H]$. In addition, there was fragmentation that loses 3 CH_3 groups and 1 H group $[M-3CH_3-H]^-$ on m/z 221 (Figure 1).

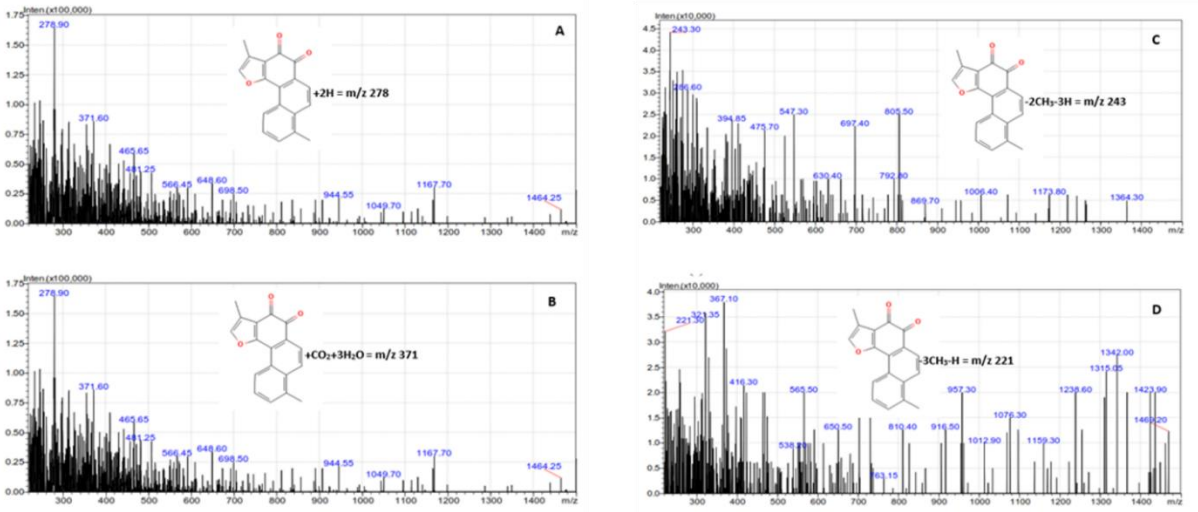

Figure 1. Fragmentation pattern of isotanshinone II
(A) Mode +ve; (B) Mode +ve; (C) Mode –ve; (D) Mode –ve.

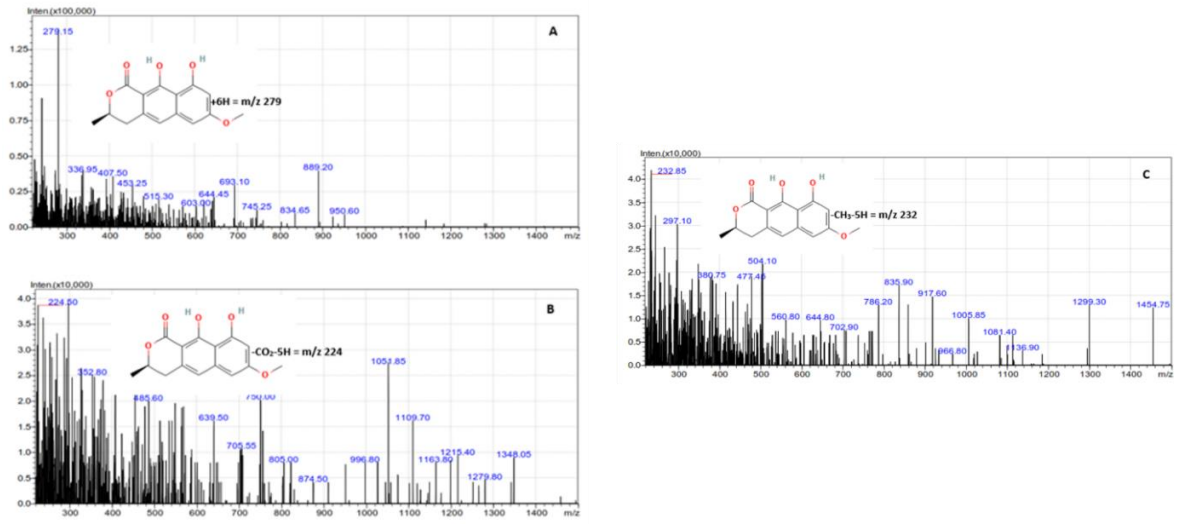

Figure 2. Fragmentation pattern of *R*-semivioxanthin
(A) Mode +ve; (B) Mode –ve; (C) Mode –ve

Furthermore, observations were also made on the MS spectrum at t_R 8.26, 9.55 and 15.48 (entries 7-9). In the positive ionization pattern, there were molecular ions with the ionization character of the flavonoid compound (*R*)-semivioxanthin (m/z = 273). Positive ion molecules were marked with the addition of hydrogen atoms $[M+6H]^+$ m/z 279 with 100% abundance percent. Then, in the negative ionization pattern, fragmentation was defined with the missing molecules of CO_2, CH_3 and hydrogen atoms. For example, in the m/z 224 molecular fragment which lost 1 CO_2 group and 5 H atoms $[M-CO_2-5H]^-$ along with the m/z 232 molecular fragment that lost 1 CH_3 group and 5 H atoms [M- CH_3-5H] (Figure 2).

Cytotoxic Activity

Cytotoxicity of the various extracts, fractions, and subfractions against T47D and Vero cells was determined using the MTT assay, which assesses cell viability through the formation of formazan. Figure 3 and Table 3 shown cytotoxic activity data from extracts, fractions, and subfractions of *E. cinereum* R.Br. The ethyl acetate extract exhibited the most potent cytotoxic activity among the extracts to T47D cells (IC_{50} 285 ± 6 µg/mL). The dichloromethane fraction of the ethyl acetate extract had the best toxic activity (IC_{50} 210 ± 11 µg/mL) of the two fractions, while SF1 had the best activity (IC_{50} 84.8 ± 7.8 µg/mL) among the subfractions though still higher compared to standard doxorubicin (IC_{50} 0.610 µg/mL). With all extracts, the toxicity to Vero cells was substantially less than to T47D cells (Table 3).

The result showed that the IC_{50} levels of the compounds decreased along with the fractionation process. The extraction of *E. cinereum* used solvents with three different polarities (*n*-hexane, ethyl acetate, and methanol) that should yield different substances. Although the IC_{50} results did not show any highly active extract, the ethyl acetate extract was considered promising for further testing because it had the lowest IC_{50} level. Fractionation of the ethyl acetate extract revealed that the dichloromethane fraction was more active than the ethyl acetate fraction and led to the sub fractionation process. The SF1 and SF2 subfractions were most active based on their lower IC_{50}s. In addition, the results against the Vero cell line indicated that the SF1 and subfractions SF2 were not only active against a cancer cell line, but also relatively inactive against a normal cell line.

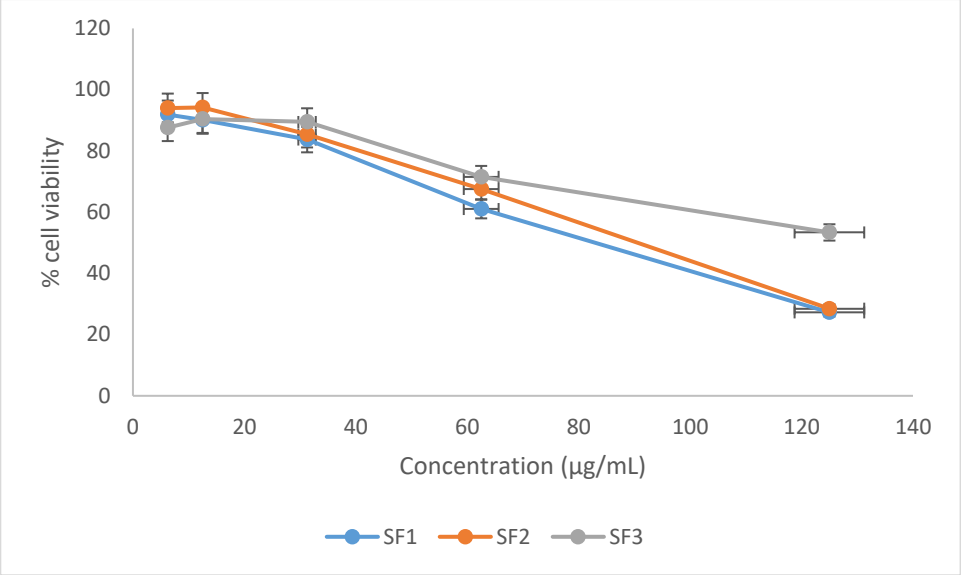

Figure 3. The graph of the concentration of subfractions SF1–SF3 of *E. cinereum* against the percentage of T47D cell viability

Table 3. Cytotoxic activity of extracts, fractions, and subfractions of *E. cinereum* against T47D and Vero cells

		T47D cell (IC_{50})[#]	**Vero cell (IC_{50})[#]**	**Selectivity index (SI)**
Extract	*n*-Hexane	>500	>500	>2
	Ethyl acetate	285 ± 6	>500	>2
	Methanol	291 ± 7	>500	>2
Fraction	Ethyl acetate	>500	>500	>2
	Dichloromethane	210 ± 11	>500	>2
Subfraction (from dichloromethane fraction)	SF1	84.8 ± 7.8	441 ± 92	5.19
	SF2	89.7 ± 3.4	303 ± 94	3.48
	SF3	137 ± 12	>500	>3.65
Doxorubicin (Control)		0.610		

[#]Data are expressed as mean µg/mL ± SD based on 3-independent assay.

CONCLUSION

The SF1 subfraction, containing terpene active compound, possessed the best cytotoxic activity against T47D cells (IC_{50} 84.8 ± 7.8 µg/mL). This subfraction also selective (tested against Vero cells) with selectivity index at 5.19 and thus has the potential to be developed as a natural anticancer therapy. However, further research focusing on purification, compound identification, and finding the mode(s) of action are important to develop *E. cinereum* extracts as potent anticancer agents.

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REFERENCES

1. WHO (2020) Cancer Profile 2020. WHO: Geneva.

2. Globocan (2018) The Global Cancer observatory. WHO: Lyon.

3. WHO (2006) EMRO Technical Publications Series 31: Guideline for management breast cancer. WHO: Egypt.

4. Aisyah, N., Andayani, T. M. and Puspandari, D. A. (2018) Analisis biaya kemoterapi pada pasien rawat inap kanker payudara peserta JKN di

RSUD Uin Banjarmasin. *Jurnal Ilmu Ibnu Sina*, **3(2)**, 333–342

5. Abotaleb, M., Kubatka, P., Caprnda, M., Varghese, E., Zolakova, B., Zubor, P., Opatrilova, R., Kruzliak, P., Stefanicka, P. and Busselberg, D. (2018) Chemotherapeutic agents for the treatment of metastatic breast cancer: An update. *Biomedicine & Pharmacotherapy*, **101**, 458–477.

6. Wang, H., Khor, T. O., Shu, L., Su, Z. Y., Fuentes, F., Lee, J. H. and Kong, A. N. (2012) Plants vs. cancer: a review on natural phytochemicals in preventing and treating cancers and their druggability. *Anti-cancer in Agents Medicinal Chemistry*, **12(10)**, 1281–305.

7. Fridlender, M., Kapulnik, Y. and Koltai, H. (2015) Plant derived substances with anti-cancer activity: from folklore to practice. *Frontier in Plant Science*, **6**, 799–808.

8. Fan, Y., Lu, H., Ma, H., Feng, F., Hu, X., Zhang, Q., Wang, J., Xu, Y. and Zhao, Q. (2015) Bioactive compounds of *Eriocaulon sieboldianum* blocking proliferation and inducing apoptosis of HepG2 cells might be involved in Aurora kinase inhibition. *Food and Function*, **6**, 12, 3746–3759.

9. Qiao, X., Ye, G., Liu, C. -F., Zhang, Z. -X., Tu, Q., Dong, J., Li, Y. -Q., Guo, D. -A. and Ye, M. (2012) Chemical analysis of *Eriocaulon buergerianum* and adulterating species by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **57**, 133–142.

10. Fan, Y., Lu, H., An, L., Wang, C., Zhou, Z., Feng, F., Ma, H., Xu, Y. and Zhao, Q. (2016) Effect of active fraction of *Eriocaulon sieboldianum* on human leukemia K562 cells via proliferation inhibition, cell cycle arrest and apoptosis induction. *Environmental Toxicology and Pharmacology*, **43**, 13–20.

11. Xu, Q., Xie, H., Wu, P. and Wei, X. (2013) Flavonoids from the capitula of *Eriocaulon australe*. *Food Chemistry*, **139**, 149–154.

12. dos Santos, L. C., Rodrigues, C. M., Silva, M. A., Coelho, R. G., Sannomiya, M. and Vilegas, W. (2005) Chemical profile of *Eriocaulon ligulatum* (Vell.) L.B. Smith (Eriocaulaceae). *Biochemical Systematics and Ecology*, **33(11)**, 1159–1166.

13. Nugraha, A. T., Ramadhan, V., Pandapotan, H. and Romadonsyah, F. (2017) A study of proliverative actvity of Herbs *Eriocaulon Cinereum* R. Br on cervical cancer cells (HeLa) with MTT assay method. *International Journal of Pharma Medicine and Biological Sciences*, **6(2)**, 73–76.

14. Lavilla, I. and Bendicho, C. (2018) *Water Extraction of Bioactive Compounds: From Plants to Drug Development*, Elsevier.

15. Veeramuthu, D., Raja, W. R. T., Al-Dhabi, N. A. and Savarimuthu, I. (2017) *Flavonoids - From Biosynthesis to Human Health*, IntechOpen.

16. Middleton, E., Jr., Kandaswami, C. and Theoharides, T. C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews*, **52(4)**, 673–751.

17. Chen, Y. C., Shen, S. C., Lee, W. R., Lin, H. Y., Ko, C. H., Shih, C. M. and Yang, L. L. (2002) Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. *Archives of Toxicology*, **76**, 351–359.

18. Madunic, J., Madunic, I. V., Gajski, G., Popic, J. and Garaj-Vrhovac, V. (2018) Apigenin: A dietary flavonoid with diverse anticancer properties. *Cancer Letters*, **413**, 11–22.

19. Saeidnia, S. (2015) *New Approaches to Natural Anticancer Drugs*. London: Springer.

20. Salvador, J. A., Carvalho, J. F., Neves, M. A., Silvestre, S. M., Leitão, A. J., Silva, M. M. and Sá e Melo, M. L. (2013) Anticancer steroids: linking natural and semi-synthetic compounds. *Natural Product Reports*, **30**, 2, 324–374.

21. Gupta, A., Kumar, B. S. and Negi, A. S. (2013) Current status on development of steroids as anticancer agents. *The Journal of Steroid Biochemistry and Molecular Biology*, **137**, 242–270.