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Research Article

Cytotoxic Effect of the Paku Atai Merah (*Angiopteris ferox* Copel) Fraction on MCF-7 and HeLa Cells and its Compound Profile by GC-MS

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Abstract

Cancer is a condition of abnormal cell proliferation of tissue cells in the body that becomes malignant. It can attack other parts of the body and affect the normal function of the body organs. The sample used in this study was tubers of paku atai merah (*Angiopteris ferox* Copel), then extracted using 96% ethanol eluent to obtain a thick extract. The ethanolic extract of *A. ferox* was fractionated using column chromatography to get the active fraction to characterize the compound using thin-layer chromatography and gas chromatography-mass spectroscopy (GC-MS) and tested its cytotoxic effectiveness on MCF-7 and HeLa cancer cells. The results of this study were obtained from fractionation using the column chromatography method to get sub-fraction C and the results of compound characterization using GC-MS and obtained variations in the class of compounds contained in the sample: amino acids, glucosinolates, alkaloids, flavonoids, and terpenoids. Based on the cytotoxic effect test of sub-fraction C on MCF-7 cells, the results obtained moderate cytotoxic effects with an IC_{50} value of 61.027 µg/mL, and HeLa cells had an IC_{50} value of 521.03 µg/mL, which was categorized as having a weak cytotoxic effect. Based on the results obtained from this study, it can be concluded that sub-fraction C of *A. ferox* tubers has a cytotoxic effect on MCF-7 cells to be used as a reference for tracing pure compounds from *A. ferox* tuber.

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INTRODUCTION

Cancer is a condition of abnormal cell proliferation of tissue cells in the body that becomes malignant. It can attack other parts of the body and affect the normal function of body organs[1-3]. Cancer is a severe problem. As many as 8.2 million cases of death are caused by cancer. Breast cancer is the first most common sufferer in the Asian region, with 23% of breast cancer[2]. Breast cancer can be characterized by a disruption in the proliferation of abnormal mammary cells that turn into malignant cells through various pathways of cell mutagenesis. One of the mechanisms of breast cancer is signal transduction of estrogen receptors (ERα and ERβ) which is a factor in activating or suppressing the expression of target genes on ligand

binding[4,5]. The ERα has a significant role of about 75% in the pathogenesis of breast cancer by promoting the growth of breast tumor cells. The ERα reacted with cyclin D1, which can activate cyclin-dependent kinases (CDKs) to change the transition of cells from the G1 phase to the S phase into cancer cells[5,6].

Various technological and scientific developments for cancer treatment have been carried out, starting from surgery, radiotherapy, chemotherapy, immunotherapy, hormone therapy, stem cell transplantation, and radiation therapy[7,8]. However, some of these therapies have various side effects: hair loss, decreased white blood cells, and decreased immune quality. The high cost of cancer treatment is not proportional to the success rate of therapy in cancer[7,9,10]. Therefore, to minimize excessive side effects on cancer treatment, several natural ingredients have been developed by looking at the cytotoxic effects of secondary metabolite compounds in plants that function as adjuvant anticancer therapy that have proliferative pro-apoptotic properties[11-14].

One of the plants with anticancer activity is paku atai merah or *Angiopteris ferox* Copel from the Marratiaceae family. The community has widely used *A. ferox*, especially in the Dayak area, Kalimantan, as a medicinal plant to treat various diseases. It is because *A. ferox* tubers contain a variety of compounds as reported in several studies by Nur *et al*[7,15,16]. Based on the results of phytochemical screening, the ethanolic extract of *A. ferox* tubers contains compounds such as flavonoids, tannins, saponins, steroids, terpenoids, phenolics, and angiopterosides. The various compounds in the *A. ferox* tubers also have antioxidant activity in reducing 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals with a strong category and iron ions with a potent category. It also has strong categories for antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nitric oxide (NO), and lipid peroxidase methods[4]. Extracts and fractions of *A. ferox* tubers have also been reported to have anticancer activity on breast cancer cells (MCF-7 and T47D), colon cancer cells (WiDr), and epithelioid cancer cells (HeLa), indicating that the ethyl fraction acetate has an effect on each cancer cell with a toxic category[2,7]. Based on the activity background as an anticancer from *A. ferox* tubers, this study focuses on tracing the active compounds with anticancer activity by isolating the active compounds from *A. ferox* tubers and then characterizing the compounds using gas chromatography-mass spectroscopy (GC-MS) and testing the cytotoxic effect on MCF-7 and HeLa cells.

MATERIALS AND METHODS

Materials

The materials used were ethanol 70% (OneMed, Indonesia), ethanol 96% (JT-Baker), silica gel 60 GF 254 (Merck, Germany), thin-layer chromatography plate (TLC, Merck, Germany), acetonitrile (JT-Baker), methanol (Merck, Germany), ethyl acetate (Merck, Germany), $FeCl_3$ (Sigma Aldrich, Germany), H_2SO_4 (Merck, Germany), phosphate-buffered saline (PBS, Gibco), penicillin-streptomycin (Gibco), sodium dodecyl sulfate (SDS, Merck, Germany), trypsin EDTA 0.25% (Gibco), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *A. ferox* Copel tuber simplicia obtained from West Kutai, East Kalimantan, Indonesia, and has been identified at the Anatomy and Science Laboratory of Universitas Mulawarman, Samarinda, Indonesia.

Methods

Sample preparation

The samples of *A. ferox* tubers collected were sorted by wet sorting, then washed under water to remove impurities still attached to the samples of *A. ferox* after the wet sorting was carried out. Then, the sample was chopped and dried by placing it in a simplicia oven at 40-60°C. Furthermore, after drying, the sample was done dry sorting and then pollinated for the extraction process.

Extraction

The extraction procedure was adopted from our previous research[2] under the same conditions. Dry simplicia as much as 1.5 kg was made into coarse powder by pounding. The coarse powder obtained was reduced in size by blending so that a

slightly coarse powder was obtained, as much as 1.2 kg. In general, simplicia powder with a larger surface area will improve the filtration because the surface of the simplicia powder in contact with the liquid filter is wider and breaks down the cell wall so that the filtered liquid can enter the cell. Simplicia powder as much as 1.2 kg was put into a tightly closed container and soaked with 96% ethanol. The simplicia was then allowed to stand for 24 x 3 hours, stirring occasionally for the first six hours, then allowed to stand for 24 x 3 hours. The filtrate was taken, the residue was re-macerated with 96%w/w ethanol. The filtrate was collected and evaporated. The viscous extract obtained was weighed, and the yield was calculated.

Thin-layer chromatography

The separation was carried out by TLC of the extract obtained to determine the eluent used in column chromatography. The extract was dissolved with the initial solvent and then spotted in the TLC and eluted with the appropriate eluent, after which it was put in a bucket and allowed to elude to the elution limit. The orientation of the eluent was carried out before separation by TLC using the ratio of methanol : ethyl acetate (9 : 1) and (8 : 2), and then one eluent was selected, which produced an excellent stain appearance with the ratio (8 : 2). Observations of the appearance of the stains were performed under UV lamps at λ of 254 and 366 nm[17].

Column chromatography

A set of column chromatography tools was prepared, then silica gel was inserted wet into the column tube. A total of 10 g of the extract was mixed using silica powder to obtain a dry powder extract. The mixture was then put into a column that already contained silica gel 60 and eluted using an eluent from non-polar to polar (*n*-hexane, ethyl acetate, ethanol with gradient concentration), starting from 100 mL *n*-hexane eluent, then further elution using ethyl acetate, and ethanol 96% with gradient concentration. The results of the obtained fractions were accommodated in a glass container. The incorporation of the fractions was carried out based on the color appearance of the solution and the stains on the TLC plate. Based on the similarity of the TLC profile, the combined fraction was then TLC to observe the spots at UV 254 nm and 366 nm. Eight fractions were obtained in the fractionation I process. The fractions were grouped according to their color and TLC profile. Fraction III (3.092 g) was then separated by column chromatography (polyamide, 60 cm x 5 cm column) using an eluent ratio of methanol : ethyl acetate (80 : 20 and 20 : 80) to obtain a sub-fraction of 7 (A-G). Sub-fraction C was characterized by compound profiles using GC-MS.

Fraction characterization

The characterization of the isolated fraction was carried out using a GC-MS to obtain the profile of the components in the fraction.

Cytotoxic assay of MCF-7 and HeLa cells

The active isolate fraction obtained was then subjected to cytotoxic testing to see the toxic effect of sub-fraction C on MCF-7 and HeLa cells using the MTT assay method following the test procedure from our previous research[4] with a slightly modified on serial concentration of sample test. The absorbance measurement of the sample using a microplate reader at a wavelength of 595 nm and the absorbance data obtained were then analyzed by looking at the percentage of cell viability and determining the IC_{50} value.

RESULTS AND DISCUSSION

Column chromatography

In this study, the sample used was *A. ferox* and then extracted using the maceration method. The maceration method was chosen because the extraction process is simple and avoids compound damage[18]. The extraction process using the maceration method uses 96% ethanol solvent to dissolve both non-polar and polar compounds so that the extraction process occurs entirely. Besides that, it avoids compound damage due to the growth of microorganisms during the process of making thick extracts of *A. ferox* tubers. The ethanol extract obtained was then fractionated by a silica chromatographic

column eluted using several solvents based on a concentration gradient. The results of column chromatography show that from the results of column chromatography, 42 fractions were obtained. The obtained fractions were combined based on the TLC color and stain profile eluted using methanol : ethyl acetate (8 : 2) in 10 mL. The merger results obtained eight fractions given each code (**Figure 1**). Fractions III were column chromatographed again with methanol : ethyl acetate (80 : 20 and 20 : 80) in 100 mL. The chromatography results obtained 35 fractions, which were then combined based on spot color and stain profile using an eluent ratio of methanol : acetone (8 : 2) in 10 mL to obtain seven fractions from the combined results. Sub-fraction C (7-11) was characterized using GC-MS to determine the profile of the compounds contained in the sub-fraction. Sub-fraction C was chosen for further characterization because the resulting spot pattern showed the presence of phenolic compounds after being sprayed using the $FeCl_3$ reagent, which formed a blue spot (**Figure 2A**).

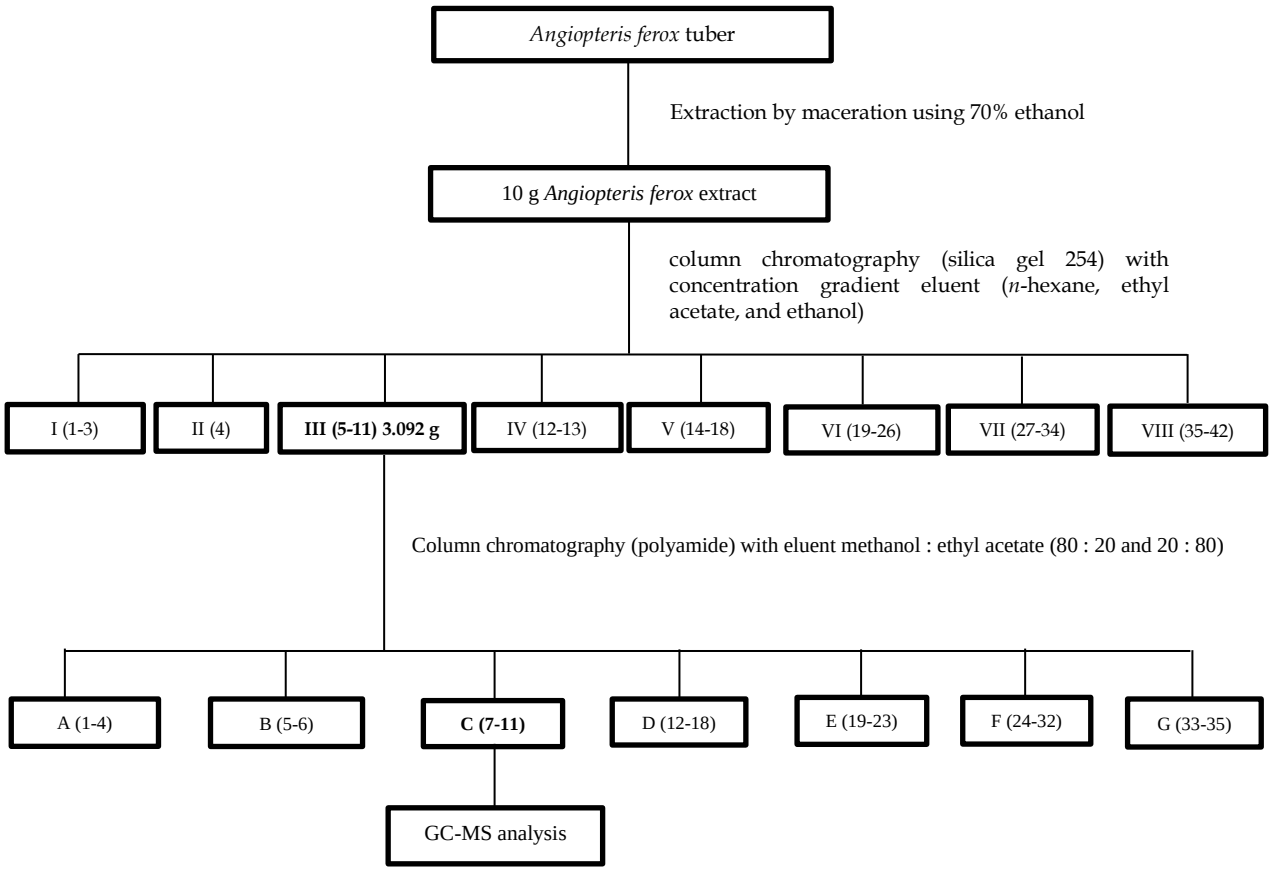
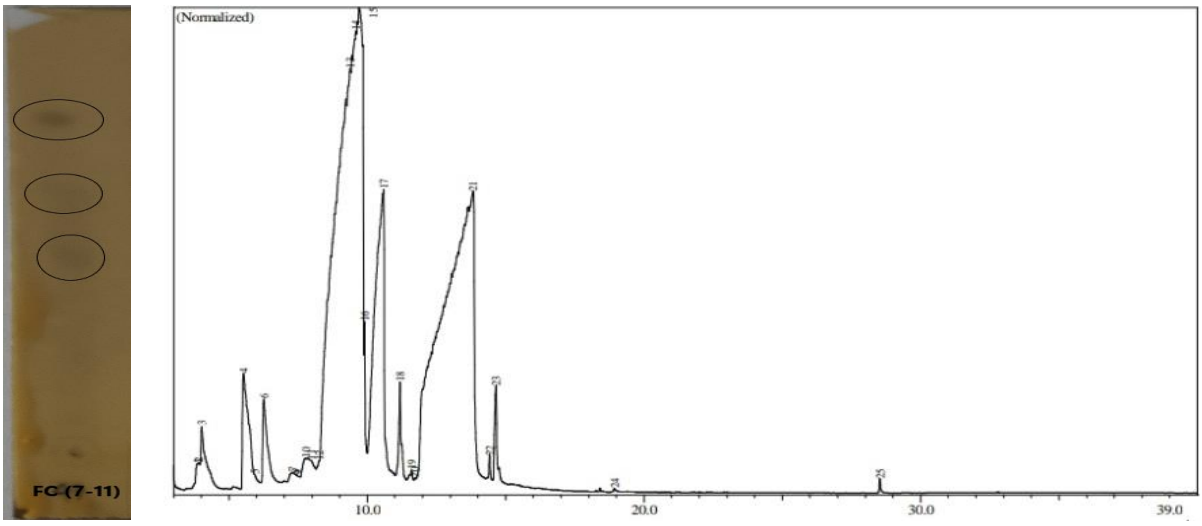


Figure 1. The process of compound fractions from the ethanol extract of *A. ferox*

Compounds characterizations

Compound characterization of sub-fraction C was carried out by looking at the profiles of the compounds found from sub-fraction using the GC-MS. The GC-MS data fragmentation (m/z) was processed using the *ReSpect for phytochemical* (http://spectra.psc.riken.jp/menta.cgi/respect/search/fragment) to see fragments that indicate the intensity of secondary metabolites contained in the isolates of *A. ferox* tubers (**Figure 2B**). Based on the GC–MS data obtained from identifying the *A. ferox* tuber isolates, it was shown that the sub-fraction C tested contained secondary metabolites, such as alkaloids, flavonoids, and terpenoids (**Table I**). Alkaloid compounds in the chromatograms obtained were indicated by peak numbers 4, 10, 14, 17, 18 and 19 in fragments 41, 42, 55, 57, 58, 68, 69, 73, 82, 84, 96, 97, 98, 101, 110, 113, 114, 129, 131 and 146. At the same time, the flavonoid compounds were shown by peaks 5, 6, 11, 22, 24 and 25 in fragments 41, 42, 57, 69, 70, 71, 73, 81, 84, 85 97, 103, 111, 129, 167, 199, 213, 256, 279 and 390. While the terpenoid compounds were shown by peaks 8, 12, 13, and 15 in fragments 55, 69, 97, and 115. The data show terpenoid compounds at peak 12-15, alkaloids at peak 17-19, and flavonoids at peak 22-25.



a **b**

Figure 2. Blue spot profile in TLC after spraying $FeCl_3$ reagent (**a**) and chromatogram of the sub-fraction C using the GC-MS (**b**)

Table I. Results of identification of compound groups using the GC-MS

No	Compounds detected	T_R (minute)	*m/z* prediction by ReSpect for phytochemicals
1	Amino acid	3.867	14 25 27 30 39 4453 55 58 84 86
2	Amino acid	3.908	14 25 27 30 39 4453 55 58 84 86
3	Glucosinolate	4.012	26 41 50 55 5766 69 7282 96 98
4	Alkaloids	5.529	18 26 31 43 54 55 61 68 82 108 110
5	Flavonoids	5.933	38 39 43 50 54 55 62 67 71 82 83 95 110 111
6	Flavanoids	6.264	28 39 41 54 68 82 110 111
7	Amino acid	7.302	39 43 55 57 85 86 128
8	Terpenoids	7.408	15 18 29 31 38 43 4655 61 69 85
9	ND	7.475	27 29 39 43 50 57 85 128
10	Alkaloids	7.790	29 41 45 54 56 63 69 73 84 85 95 98 113 131
11	Flavonoids	7.975	15 29 41 43 56 58 69 81 85 103 129 143 157
12	Terpenoids	8.258	29 39 45 55 58 69 86 87 97 115
13	Terpenoids	9.381	29 39 45 55 58 69 86 87 97 115
14	ND	9.587	27 41 42 51 58 6070 82 86 98 129
15	Terpenoids	9.702	29 39 45 55 58 69 86 8797 115
16	ND	9.905	39 45 57 85
17	Alkaloids	10.596	15 27 39 50 55 68 84 96 114
18	Alkaloids	11.183	15 29 41 44 57 69 74 86 97 146
19	Alkaloids	11.603	18 29 41 53 59 61 69 73 84 85 101 114
20	ND	11.725	28 30 41 44 56 66 78 84 88 99 115 143
21	ND	13.832	47 55 60 71 74 84 89100 118 160
22	Flavanoids	14.425	15 27 29 42 57 60 70 73 85 97
23	ND	14.652	29 33 42 4656 60 63 74 75 102 105 132
24	Flavonoids	18.939	29 43 57 73 85 98 111 129 143 157 171 185 199 213 227 239 256
25	Flavanoids	28.533	27 41 57 71 84 104 113 132 149 167 168 261 279 280 390

ND: compound not determined

Cytotoxic assay

Evaluation of the cytotoxic effects of the A. ferox tuber fraction using the MTT assay method on MCF-7 and HeLa cells was performed to evaluate the potential of the A. ferox tuber fraction in inhibiting cell proliferation with percent cell viability and toxic effect based on IC_{50} value. The IC_{50} value is a concentration value required for a sample to give a toxic effect of 50% on cells categorized as strong cytotoxic effect <50 µg/mL, moderate cytotoxic effect 50-200 µg/mL, weak cytotoxic effect 200-1,000 µg/mL and no cytotoxic effect >1,000 µg/mL. The cytotoxic effectiveness test of the *A. ferox* tuber fraction (**Figure 3**) showed that the *A. ferox* tuber fraction had a moderate cytotoxic effect on MFC-7 cells with an IC_{50} value of 61.027 µg/mL. Meanwhile, the *A. ferox* tuber fraction had a weak cytotoxic effect in HeLa cells with an IC_{50} value of >500 µg/mL.

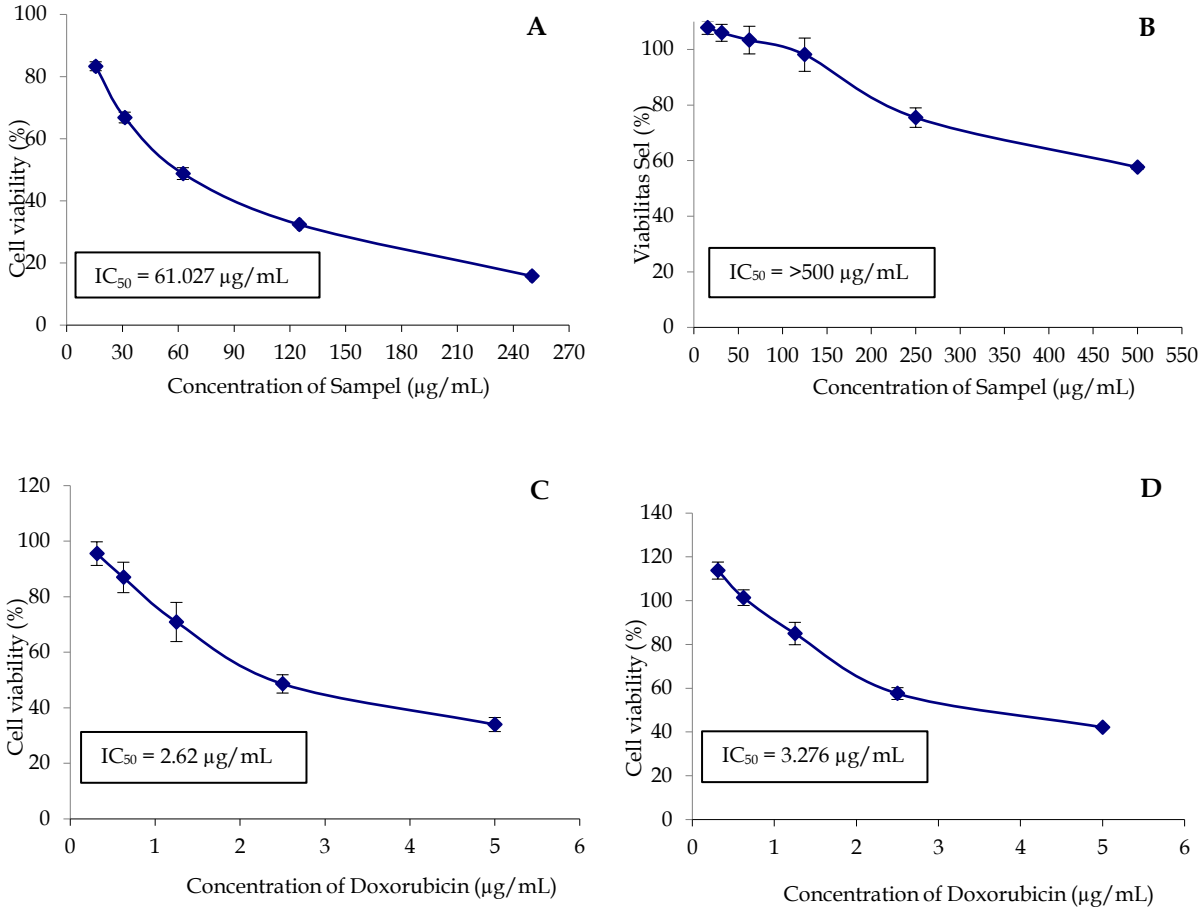


Figure 3. The graph of cytotoxic activity of sub-fraction C of *A. ferox* tuber toward MCF-7 (**A**) and HeLa cells (**B**) and doxorubicin as positive control toward MCF-7 (**C**) and HeLa cells (**D**). The data were observed in triplicate (n=3)

Meanwhile, the IC_{50} value of doxorubicin positive control against MCF-7 and HeLa cells obtained an IC_{50} value of 2.62 and 3.276 µg/mL, respectively, and included in the strong cytotoxic category. This study showed that sub-fraction C of *A. ferox* extract had a toxic effect on MCF-7 but not on HeLa cells. This mechanism is influenced by compounds' content in the sub-fraction of *A. ferox*, which could not cause apoptosis in HeLa cells. The sub-fraction C of *A. ferox* tubers has activity on MCF-7 cells based on the analysis of compound groups using GC-MS containing several compounds (**Table I**). According to previous research[13,19], phenolic compounds can inhibit the formation and growth of tumors by inducing cell cycle arrest and undergoing cell apoptosis. Phenolic compounds can induce cell cycle arrest with multiple cell cycles from G1-S-G2 so that they can downregulate cyclins and CDKs, and directly induce gene expression in p21, p27, and p53. According to other studies[20-22], flavonoid compounds have the potential as pro-oxidants so that they can suppress the proliferation of cancer cells by inhibiting the epidermal growth factor receptor or mitogen active protein kinase (EGFR/MAPK), phosphatidylinositide 3-kinases (PI3K), protein kinase B (Akt), and nuclear factor-kappa-β (NF-kB)[23].

CONCLUSION

Based on the results obtained from this study, it can be concluded that sub-fraction C of *A. ferox* tubers has anticancer activity, which was tested using an MTT assay on MCF-7 cells with an IC_{50} value of 61.027 µg/mL in the moderate toxic effect category. This result occurs because the sub-fraction C results from the compound groups' characterization using GC-MS. Several compounds are obtained, i.e., amino acids, glucosinolates, alkaloids, flavonoids, and terpenoids, to have a toxic effect on cancer cells.

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AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The author declares there is no conflict of interest.

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