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

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 IC₅₀ value of 61.027 µg/mL, and HeLa cells had an IC₅₀ 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.

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 the body organs. 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. 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. 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.

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. 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. 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.

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. 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 angiopteroids. 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-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nitric oxide (NO), and lipid peroxidase methods. 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. 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), acetonitrile (JT-Baker), methanol (Merck, Germany), ethyl acetate (Merck, Germany), FeCl₃ (Sigma Aldrich, Germany), H₂SO₄ (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 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.

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 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 IC50 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. 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 FeCl₃ 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, 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.
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 IC₅₀ value. The IC₅₀ 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 MCF-7 cells with an IC₅₀ value of 61.027 µg/mL. Meanwhile, the A. ferox tuber fraction had a weak cytotoxic effect in HeLa cells with an IC₅₀ value of >500 µg/mL.

Table 1. Results of identification of compound groups using the GC-MS

<table>
<thead>
<tr>
<th>No</th>
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<th>Ts (minute)</th>
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<tr>
<td>1</td>
<td>Amino acid</td>
<td>3.867</td>
<td>14 25 27 30 39 4453 55 58 84 86</td>
</tr>
<tr>
<td>2</td>
<td>Amino acid</td>
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<td>14 25 27 30 39 4453 55 58 84 86</td>
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<td>38 39 43 50 54 55 62 67 71 82 83 95 110 111</td>
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<td>6</td>
<td>Flavanoids</td>
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</tr>
<tr>
<td>7</td>
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<td>Flavanoids</td>
<td>28.533</td>
<td>27 41 57 71 84 104 113 132 149 167 168 261 279 280 390</td>
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</table>

ND: compound not determined
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), phosphatidylinositol 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.
ACKNOWLEDGMENT

We want to thank the Indonesian Government, the Ministry of Education, Culture, Research, and Technology, for the funding provided through a higher education cooperation grant with contract No. 01/A/BAST/2021 and 0397/E.E4/PT.01.02/2021.

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.

REFERENCES


