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In vitro antioxidant and cytotoxic evaluation of ethyl acetate fraction of angiopteris ferox copel tuber against htb lung cancer cell

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Abstract

The tubers of *Angiopteris ferox* Copel from the Mirrataceae family have been widely used empirically by society for various treatments. This experimentation aimed to discover the antioxidant and anticancer action of the ethyl acetate (EA) fraction of *Angiopteris ferox* Copel tubers against HTB lung cancer and normal cells in vitro. The EA-fraction obtained from the liquid-liquid extraction was tested for antioxidant activity using the beta carotene degradation and the hydroxyl radical method. The EA-fraction tries the cytotoxic sequel on lung cancer and normal cells using the MTT test method. The outcome achieved that the EA-fraction of the sample has antioxidant activity in reducing lipid peroxidation radicals with an IC_{50} grade of 30.29 µg /mL (extreme category) and hydroxyl radicals with an IC_{50} grade of 126.62 µg /mL (medium category). In cytotoxic activity has a medium toxic effect on pulmonary HTB cell line with an IC_{50} grade of 78.96 µg /mL. In comparison, normal Vero cells have a feeble harmful impact with an IC_{50} rate >500 µg /mL. It could have culminated that the EA-fraction of *Angiopterix ferox* Copel has antiradical activity and has a toxic effect on HTB cancer cells but is weak against normal Vero cells. It shall promote as a remedy for the management of lung cancer.

Keyword : *Angiopteris ferox* Copel; Antioxidant; Cytotoxic; HTB cells; Vero Cells

1. Introduction

World Health Organization inform that, in 2018, lung cancer was the highest cancer case in men with a percentage of 14.5% and the third highest in women after breast cancer and colorectal cancer with a portion of 8.4% (WHO, 2020). Cancer is a form of uncontrolled cell proliferation and is a substantial fitness worry widespread. Cancer is the third prominent bring of death after cardiac disease and stroke[1–3]. Lung cancer is either of the most prevalent occurrences, causing more deaths than other cancers [4], [5]. Free radicals can cause cancer due to vulnerability to UV radiance or carcinogens, causing a quick expansion in reactive oxidative stress. These terrible oxidative stress environments induce immedicable carcinogenesis [6].

Several medical treatments (radiotherapy, chemotherapy, and surgery) and non-medical (traditional medicine) have developed. Medical treatment can provide side effects such as bleeding, skin problems, hair loss, nausea and vomiting, memory problems, and several other side effects [7–9]. On the other hand, phytochemical compounds have a function as antiproliferative and proapoptotic effects [6], [10], [11]. Therefore, traditional plants or bioactive compounds are continuously developed with anticancer activity to minimize side effects, such as natural herbal medicines [10], [12].

Indonesia has a diversity of traditional plants that are used empirically as medicine. One of the conventional plants used as medicine is *Angiopteris ferox* Copel tuber from the Marratiaceae family. *A.ferox* Copel has been considerably used empirically by the community in various treatments. This plant has substances alkaloids, flavonoids, phenols, tannins, and saponins [13]. In addition, [14] isolated secondary metabolites, namely Angiopteroside. Several studies have shown that the *Angiopteris ferox* Copel in ethyl acetate fraction has bioactivity as an antioxidant carried out using the DPPH, ABTS, and FRAP methods [13]. In addition, the ethyl acetate fraction has a powerful toxic effect on shrimp larvae

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[13] and a moderate harmful effect on cancer cells [12].

This study aims to describe the cytotoxic effects of the *Angiopreris ferox* Copel plant on HTB lung cancer cell lines that have not been previously reported. In addition, several studies also conducted several research activities as an inhibitor of oxidative stress as supporting data. The end of this work is intended to provide information related to the bioactivity of *Angiopteris ferox* Copel. Investigators can use it for development in the discovery of new drug compounds.

2. Experimental

2.1 Materials

The materials used include aluminum foil, aqua dest (One Med®), DMSO (Dimethyl Sulfoxide) (Merck, Germany), linoleic acid p.a (Aldrich), ethanol 96% (Bratachem), ethyl acetate (Merck, Germany), DMEM (eagle media modified by Dulbecco, Sigma Aldrich) media, FBS (Fetal Cow Serum, Sigma Aldrich), HEPES buffer media (Sigma Aldrich), chloroform (German brand), L-Glutamine, MTT (3- (4,5-dimethyl-thiazol-2-il-2,5-diphenyltetrazolium bromide, Gibco®), n-Hexane (Merck, Germany), PBS (Phosphate Buffered Saline, Gibco®), Penicillin-streptomycin (Gibco®), SDS (Sodium dodecyl sulfate) (Merck, Germany), trypsin EDTA 0.25% (Gibco®), hydrogen peroxide (H_2O_2), HCl, *Angiopteris ferox* Copel Tuber, β-carotene (Japanese TCI), Tween 20 p.a (Merck, Germany), lung cancer HTB cells and normal Vero cells obtained from In Vitro Cell Culture Laboratory of Medicine and Health Science Faculty, Universitas Muhammadiyah Yogyakarta.

2.2 Sample Preparation

The samples in this study are tuber from Angiopteris ferox Copel obtained from Linggang Bigung District, West Kutai Regency, East Kalimantan. We have identified the species in the Laboratory of Plant Anatomy and Science, University of Mulawarman, Indonesia. The tuber of *A.ferox* Copel collected and then cleaned under running water and cut into small pieces. Then the sample was withered in an oven at 40^o C 3 times 24 hours until the moisture content was less than 10%. The dried sample was then pollinate for the extraction process.

2.3 Extraction and Fractionation

A total of 1.2 Kg of dry sample powder was put into the extraction container and soaked, accompanied by 96% ethanol to the tune of 6.5 L for 3times 24 hours while stirring occasionally. The filtrate and excess were disparate, and the mess was re-extracted by the same process. The strainer acquired was then undiluted to use a spin vacuum evaporator until a thickset extract developed.

The crude extract was fractioned to obtain the ethyl acetate fraction. Ten grams of the natural extract was dissolved with an aqueous-ethanol (9:1) solvent mixture and put into a separating funnel. The mixture was fractionated with n-hexane solvent, aiming to separate the remaining fat in the crude extract. Then the n-hexane phase and the residue are discredited. Several times, the separation process was performed by applying n-hexane as a solvent until the n-hexane phase was clear. Then, the residual was re-fractionated using ethyl acetate, and the fractionation process using ethyl acetate in the same manner as the n-hexane solvent. The ethyl acetate liquid fraction was vaporize using a rotary vacuum evaporator to obtain ethyl acetate fraction (EAF). EAF was then testing for bioactivity as an antiradical and cytotoxic on lung cancer cell line.

2.4 Hydroxyl Radicals Assay

Testing the antioxidant activity of the ethyl acetate fraction in reducing hydroxyl radicals was carried out based on the method of [15] with a slight modification. Hydroxyl radicals are initiate from the reaction of Fenton between $FeSO_4$ and H_2O_2. The sample performed the investigation by setting up a reaction fusion conceiving 1 mL of Ferri (II) Sulphate 1.5 mM, 0.5 mL Hydrogen Peroxide 6 mM, and sodium salicylate 20 mM. Furthermore, each concentration of the sample solution (10-1000 µg / mL) and quercetin as a positive control (10-100 µg/mL) was adding. The mixture kept the fusion for 30 minutes at 37ºC. After the incubation period, the absorbance was quantifying by UV-Visible spectrophotometry at 520 nm. Determination of antioxidant action by the following equation:

Hydroxyl Radical Inhibition (%) = [1 - (A_1 – A_2)/A_0] x 100. A_0 shows the absorbance of the reagent only, A_1 shows the absorbance of the EA-fraction or positive control, and A_2 shows the absorbance in the absence of reagents (sodium salicylate).

2.5 *β– Carotene Degradation Assay*

The antioxidant activity of the ethyl acetate fraction of *A. ferox* in reducing lipid peroxidation radicals was carried out by the β-Carotene Degradation (BCD) assay [19]. Emulsion of beta carotene contains 5 mg of beta carotene pollen and dissolving with chloroform. Four mL of polysorbate 20 as surfactant and 0.5 mL of linoleic acid as peroxide radical originator (chloroform in the mixture was evaporating), the volume was adequately upset into distilled water to 250 mL and swing until a transparent emulsion of beta carotene was obtaining. A series of ethyl acetate fractions (10-1000 µg / mL)

made in the flask and 2 ml of the β-Carotene emulsion was appending. Furthermore, the mixtures' volume was added with distilled water to 5 mL in a volumetric flask and incubated in an oven for 20 minutes at 50ºC. After the incubation period, the absorbance quantifying by a spectrophotometer (461 nm). The mixtures monitored measurements for 0-120 minutes at 30 minutes intervals. The antioxidant effect was deliberated based on the dissimilarity in the reduction rate of the sample and control (β-carotene emulsion only). The samples calculated the percentage (%) of inhibition of β-carotene reduction rate based on the following formula [16]. Inhibition of Degradation Rate:

$$(\%) = [(\mathrm{Ln}\ (a/b)\ x\ 1/t\ ]\ x\ 100$$

2.6 *Oxidative Stress-induced H_2O_2*

The procedure of antioxidant activity using of Human Dermal Fibroblast adult (HDFA) cell-induced by hydrogen peroxide (H_2O_2) was carried out based on [17] with modification. The ethyl acetate fraction solution was made by 10 mg of the fraction and diffused with 100 µL of DMSO, and the capacity was sufficient into 1 mL with DMEM (10000 µg/mL). It made the concentration series of the sample from 500 to 31.25 µg/mL. A total of 100 µL of hydrogen peroxide (H_2O_2 1000 µM) was used as an oxidative stress-inducing agent. An amount of 0.1 mL (2 x 10^4 cells/well) of the cell was dispensed into 96-well plates then kept for 24 hours at a CO_2 incubator at 37º C so as the cells stick fast to the wells and added divers concentration series of samples to the medium of culture. The well plates were kept for 24 hours in a CO_2 (5%) incubator at 37ºC, thrown out the culture medium after an incubation interval. The well plate cleansed cells with 0.1 mL of phosphate-buffered saline. A sum of 0.1 mL of H_2O_2 (1000 µM) was attached to each well, then kept in a 5% CO_2 incubator for 2 hours, at 37ºC. Afterward, thrown away the medium and rinsed the cells with 100 µL of media. Add to every well with MTT reagent (0.1 mL) in PBS and kept into the CO_2 incubator at 37ºC for four h. The well plate was added with SDS 10% and then held one night till the reaction stopped. The absorbance was scan with a microplate reader at 595 nm.

2.7 Cytotoxic assay against HTB cell and Vero cell

2.7.1 Isolation and Cell Harvest

According to [18],[19], cell isolation and culture were carried out with slight modifications. The HTB cells and Vero cells obtained from In Vitro Cell Culture Laboratory of Medicine and Health Science Faculty, Universitas Muhammadiyah Yogyakarta, were cultured on a complete DMEM (Dulbecco's modified eagle medium). Cell growth observes, and every two days replaced the media with entire DMEM media. After HTB cell confluent surface about> 80%, harvest the cells. Cells are attached to the plate and washing with DMEM media. Furthermore, rinsed the layer of cells attached to the dish using the trypsin-EDTA solution to remove the cells from the bottom of the plate and incubated at 37ºC for 5 minutes, observed until the cells no longer stuck to the dish and expanded on the media. Added 5 mL of complete media to activate trypsin, and then the suspension was transferred to the centrifugation tube and centrifuged for 10 minutes at a speed of 1000 rpm. Then rejected The supernatant from the tube and the resuspended residue (cells) in 1 mL of whole media [18]. A total of 10 µL of cells were collected and transferred into a hemocytometer, and the cells were sum up using a microscope. Several cells must be conveyed into sterile tubes and added with the culture medium based on the hoped-for concentration.

2.7.2 Cytotoxic test by MTT

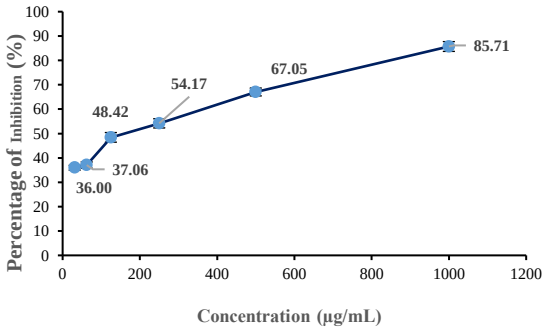
They distributed the harvested cells (2 x 10^4 cells/well) to the wells according to the number of samples tested, positive control, and negative control. Then the mixture was kept in an incubator CO_2 at 37ºC for twenty-four hours to adjust and stick to wells until cells were ripe to be managed. In the following sitting period, took the well plate containing the cell from the incubator, and then the media was discarded. Insert each 100 µL concentration series of the sample solution into well-containing HTB cells, then kept the plate for twenty-four hours into the CO_2 incubator at 37ºC. In the wake of incubation season, thrown out the cell media and a total of 0.1 mL, the MTT reagent was distributed into the well plate. The well plate was kept for four h. Then the well plate was added with a stopper reagent (100µL of SDS) and was kept overnight then, and the absorption was scanned at 595 nm with a plate reader.

3. Results and Discussion

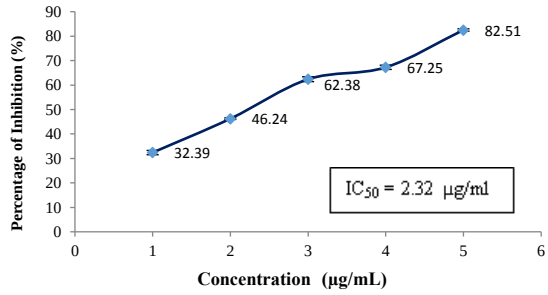
3.1. Hydroxyl radical testing

The principle of reducing hydroxyl radicals is the Fenton reaction mechanism, namely the reaction between Fe^{2+} and H_2O_2 to produce hydroxyl radicals. The hydroxyl radicals formed react with salicylates become 2,5-dihydroxy-benzoic-acid and 2,3-dihydroxy-benzoic-acid was measured by

spectrophotometer.



(A)



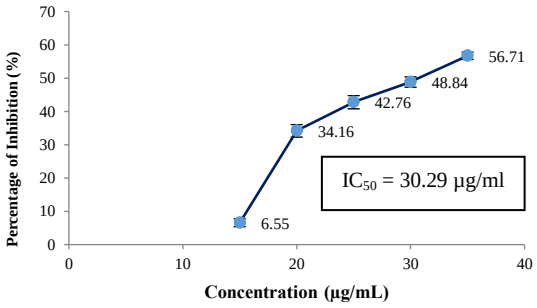
(B)

Fig 1. Graph the relationship between sample concentration and percent inhibition of ethyl acetate fraction (A) and standard quercetin (B). The data with triplicate (n=3).

The research results in reducing hydroxyl radicals show that the supreme level in the ethyl acetate fraction, the greater the strength of inhibition (**Figure 1).** Significant effects occur at the highest concentration, 1000 µg/ml, with a percent inhibition value of 85.70%. Inhibiting hydroxyl radicals up to 50% (IC_{50}) required a concentration of 126.62 µg/ml (100-150 µg/ml), a moderate level of antioxidant power, and the IC_{50} value of quercetin as a comparison was 2.32 µg/ml (<50 µg/ml) extreme antioxidant levels [19].

3.2 β- Carotene Degradation Assay

In the antioxidant activity test by the beta-carotene method of linoleic acid form free radicals from hydroperoxides produced by linoleic acid. Free radicals form from linoleic acid oxidation due to the reduction of the hydrogen atom from one diallyl methylene group, which strike out the double bond in beta carotene, causing beta carotene oxidation which causes the loss of the chromophore group, which gives the orange color [20].



(A)

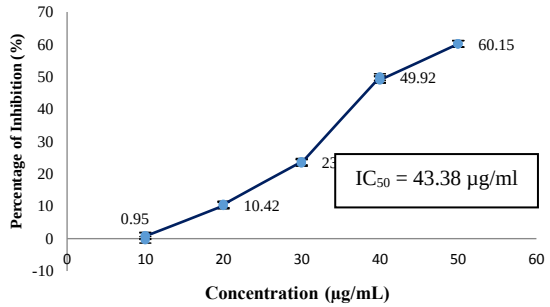


Fig.2 Graph of inhibition percentage and sample concentration of EA-fraction (A) and positive control of quercetin (B). The data with triplicate (n=3).

Antioxidant activity testing using the BCD method can be determined q… IC_{50} = 126.62 µg/ml …C_{50} value. The IC_{50} valu… …ch concentration a sample needs to inhibit 50% of radicals. Antioxidant capacity shows the smaller of IC_{50} value, the greater the potential sample in reducing free radicals. The results obtained from this study can be seen in Figure 2. The ethyl acetate fraction can reduce the color degradation of β-Carotene with an IC_{50} grade of 30.29µg/mL, contrasted to the quercetin with an IC_{50} rate of 43.38µg/mL. From the data, the IC_{50} value of the sample has almost the same potential as a positive control of quercetin. It is due to many flavonoids, and phenolic contents in samples studied [21].

3.3 Antioxidant Activity by H_2O_2-induced Oxidative Stress Method

Changes in cell morphology are a sign of cytotoxic activity resulting from a compound after treatment of cells compared to control cells. Antioxidant activity against H_2O_2-induced cells from samples using normal HDFa cells can also observe microscopically to see the morphology of control HDFa cells, as shown in Figure 3A. HDFa cell morphology after H_2O_2 treatment (3B) Observation results of HDFa cells showed differences in cell morphology before and after the MTT reagent.

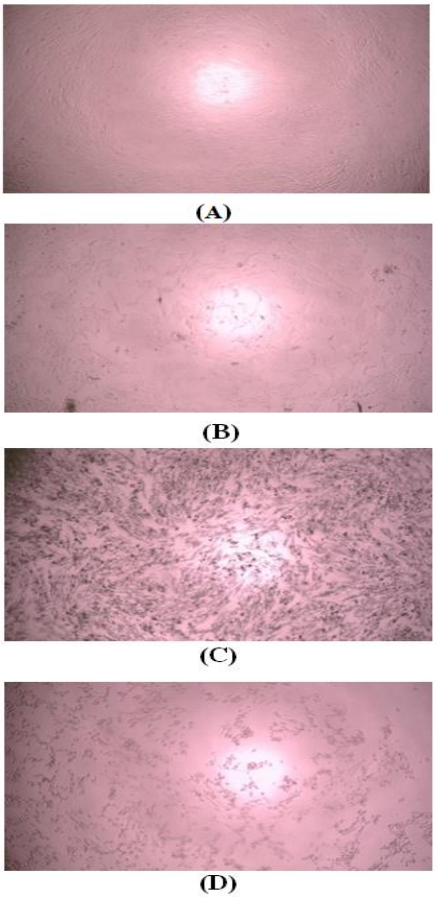


Fig. 3. Morphology of cells after treatment with MTT reagent (A) Control Cells, (B) negative control (H_2O_2 exposure without sample), (C) Cells after sample treatment (high concentration of 500 µg/mL) and H_2O_2 exposure (D) Cells after sample treatment (low concentration of 31.25 µg/mL) and H_2O_2 exposure.

In Figure 3C, more formazan crystals formed, while in Figure 3D, very few formazan crystals formed. These express that the higher the test sample level, the more formazan crystals created, meaning more cells were alive. On the other hand, exposure to H_2O_2 (1000 µM) causes cell death up to above 50%, characterized by less formazan formation. It occurs because H_2O_2 is a source of oxidative stress from an increase in Reactive Oxidative Species (ROS), which causes cell toxicity [22–24]. Therefore, the EA-fraction of a sample can prevent cells from experiencing oxidative stress after exposure to H_2O_2 so that cells are still viable. Living cells have the mitochondrial reductase enzyme, which can react with the MTT reagent to indicate a purplish-blue formazan salt formation.

Table 1. Cell viability (%) of EA-fraction of *A.ferox* Copel *by H_2O_2*-induced Oxidative Stress Method

Sample	Viability of cells (%) with level (µg/mL)					IC_{50}
	31.25	62.5	125	250	500	
Control cell			100			
Negative Control (H_2O_2)			40.45±2.25			
Ethyl Acetate Fraction	30.18±0.67	30.57±1.42	38.11±1.58	65.49±3.89	89.05±1.42	121.20
Positive Control (quercetin)	69.31+4.23	68.41±3.00	81.23±2.95	115.51±1.3	112.76±5.67	<30

The data were expressed as mean±SD, with triplicate (n=3)

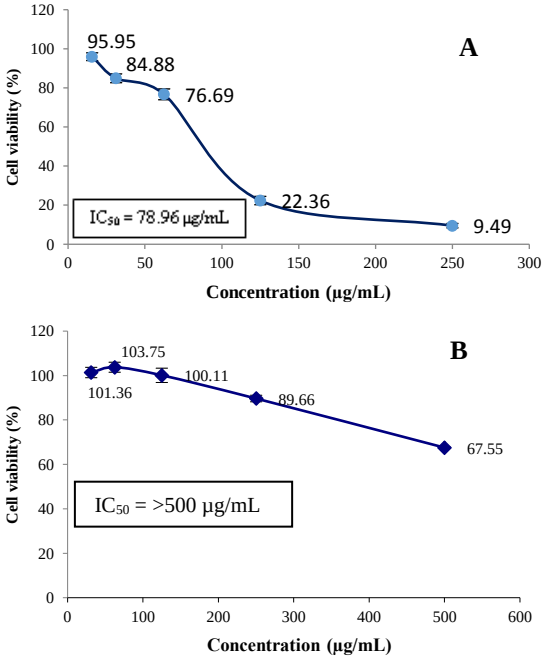
Activity testing using the oxidative stress method was carried out colorimetrically by observing the formation of formazan and measuring its absorption at 595 nm. The potential of EA-fractions compared with negative control and control cells. The resulting absorbance and the intensity of the color formed are comparable to the number of viability cells. The darker the purple color produced and the greater the absorbance value, the more cells are alive, but more cells are dead if the color is yellow. Table 1 shows that the ethyl acetate fraction of *A.ferox* Copel gives a higher percentage of cell viability with increasing concentration. At the highest concentration (500 µg/mL) was able to maintain cell viability up to 89.05±1.42 %. The test results show that the IC_{50} value of the ethyl acetate fraction was 121.20 µg/mL. The result indicates that a 121.2 µg/mL level of ethyl acetate fraction could inhibit 50% of cell mortality after exposure to H_2O_2. It shows that the ethyl acetate fraction can be in the moderate category (100-150 µg/ml) to prevent cell death due to oxidative stress.

3.4 Cytotoxic activity test with MTT

The cytotoxic evaluation of the ethyl acetate fraction of *A. ferox* Copel on lung cancer (HTB) cells to observe the potential of the sample to exert a toxic effect on cells with various series of sample concentrations. In addition, cytotoxic testing of the fraction also carrying out on normal Vero cells aims to evaluate the safety of the sample so that it has a selective effect on normal cells. It hopes that the *A.ferox* Copel fraction has a toxic outturn on HTB cancer cells despite not Vero cells developing as a supporting agent for cancer therapy. In a quantitative cytotoxicity test, an IC_{50} value was necessary, which indicates the concentration required to prevent the accretion of HTB lung cancer cells by 50% of the total population. According to [17] an IC_{50} grade <50 µg/mL is classified as potent effect, if a grade of 50

µg/mL - <20 µg/mL is classified as average, and a grade of >200 - <1000µg/mL is classified as poor effect. And the IC_{50} value >1000 µg/mL had no cytotoxic effect. It studied the determination of the IC_{50} grade according to the relationship between concentration and absorbance of the sample.

Figure 4 showed the EA-fraction of cytotoxic test results against HTB lung cancer cells and Vero normal cells. The graph in Figure 4 shows a trend that the higher the concentration, the lower the viability of cells. It indicates that the fraction has a toxic effect on cells. The ethyl acetate fraction of *A.ferox* Copel has a poisonous impact on HTB lung cancer cells with a medium category (IC_{50} grade of 78.96 µg/mL). In contrast, Vero normal cells had a weak category toxic effect (IC_{50} grade of >500 µg/mL). In this work, doxorubicin (Figure 4C), as an anticancer agent, is exerted to heal numerous kinds of cancer as though breast cancer, leukemia, bone cancer, lung cancer, and ovarian cancer. The analysis results show that doxorubicin has an IC_{50} value of 3.275 with a potent category. Based on the outcomes of this research, it showed that the ethyl acetate fraction of *A.ferox* Copel had moderate cytotoxic activity and was slightly selective against normal cells because it had a weak toxic effect. The cytotoxic effect of the EA-fraction that has been obtained has similarities with the research conducted by Aisyah et al., 2021 using a different cancer cell line, namely the T47D cell line [12].



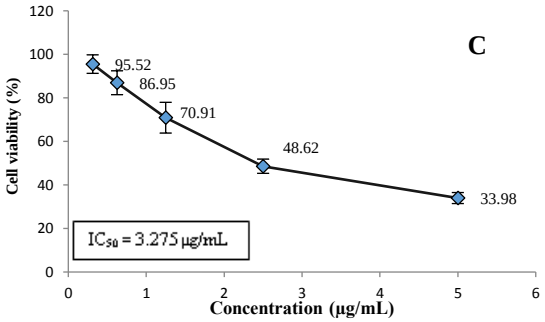


Figure 4. The graph of the cytotoxic effect of the *A.ferox* Copel fraction on cells. (A) HTB lung cancer cells, (B) Vero normal cells, and (C) cytotoxic effects of doxorubicin as a positive control against HTB lung cancer. The data with triplicate (n=3).

The presence of phenolic or flavonoid compounds in the sample may influence cytotoxic activity with the moderate category of EA-fraction against HTB lung cancer cells [13]. Several studies have reported on compounds with anticancer properties such as phenolic or flavonoids and saponins. Natural phenolic has been declared to lead cell cycle inhibition at distinct cell stages: G1, G2, S, and S-G2 by immediately adjusting cyclins-dependent-kinases (CDKs) or implicitly inducing gene expressions p21, p27, and p53. Also, several studies have shown that natural phenolic indicate different effects on cancer cells than normal cells [25]. Flavonoids play a role in cancer cells by inhibiting MAPK in the receptor signaling pathway like tyrosine kinase. Flavonoid also has a role in inactivating proteins that present a bit part in transduction signal and culminate in the blockade of growth factor receptors. Flavonols take part a role in targeting cell surfaces of enzyme transduction signals, like a protein tyrosine kinase, adhesion focal kinase (AFK), and angiogenesis processes [10], [26].

4. Conclusions

The bioactivity test of the EA-fraction of *Angiopteris. ferox* Copel on its antioxidant power and toxicity to lung and normal cell line has been done. The results show intercourse in the middle of antioxidant activity and its toxicity effect on lung cancer cells. The moderate impact on lung cancer cells and the weak effect on normal Vero cells allowed the EA-fraction of *Angiopteris ferox* Copel to be further investigated for the secondary metabolites contained in it to provide a more potent effect.

5. Conflicts of interest

Based on this research, it stated that there is no conflict of interest

6. Acknowledgments

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