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Antioxidant Properties and Cytotoxic Activity of Ethyl Acetate Fraction of *Plectranthus amboinicus* (Lour.) Spreng. Leaves on HeLa and T47D Cell Lines

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Abstract

Research into plants with anticancer effects is actively encouraged in order to discover new drugs with lesser toxicity but more potent effects. The aims of study are to evaluate the antioxidant properties and to investigate the cytotoxic activity of *Plectranthus amboinicus* (Lour.) Spreng. leaves ethyl acetate fractions on HeLa,T47D and MCF7 cell lines. The extract was prepared by graded maceration using n-hexane and ethyl acetate. The ethyl acetate extract was fractionated in vacuum liquid chromatography with n-hexane: ethyl acetate; and ethyl acetate: methanol as mobile phase. Then, the fractions were analyzed with thin layer chromatography (TLC). The free radical scavenging activity was measured by DPPH method, the total flavonoid content was calculated by quercetin equivalent and the absorbance is measured by using UV-Visible spectrophotometry. The cytotoxic activity were determined using MTT assay. The fractions contained 5 sub fractions with same TLC profile. The fractions showed antioxidant activity by DPPH method with different IC_{50} values, namely: 130 µg/mL(I), 127 µg/mL(II), 137 µg/mL(III), 129 µg/mL(IV), and 124 µg/mL(V), respectively. The measurement of total flavonoid content showed 118 mg QE/g (I), 50 mg QE/g (II), 207 mg QE/g (III), 56 mg QE/g (IV), and 55 mg QE/g (V). The IC_{50} of each sub fractions on HeLa cell were 77 µg/mL, 46 µg/mL, 93 µg/mL, 71 µg/mL and 476 µg/mL; for T47D cell were 1621 µg/mL, 111 µg/mL, 128 µg/mL, 150 µg/mL and 209 µg/mL; and for MCF7 were 259 µg/mL, 343 µg/mL, 575 µg/mL, 408 µg/mL and 250 µg/mL. Based on the results, the fractions derived from ethyl acetate extract of *Plectranthus amboinicus* (Lour.) Spreng. leaves exhibit antioxidant. The Fraction II from ethyl acetate extract of *Plectranthus amboinicus* (Lour.) Spreng. was the most cytotoxic on HeLa, T47D and MCF7 cell lines. It is potential to undergo further isolation of its cytotoxic compounds.

Keywords : *antioxidant, cytotoxic, Plectranthus amboinicul (Lour.) Spreng., ethyl acetate fractions*

INTRODUCTION

The use of medicinal plant extracts for the treatment of human disease is an ancient practice that has been significantly increasing in recent years. Free radicals trigger the degenerative disease such as cancer. Cancer has become one of the most prevalent and distressing disease with increasing sufferer in the last 50 years (Asrin,

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et al., 2017; Dalimunthe, *et al*., 2018). The free radicals can be neutralized by antioxidant from intracellular (endogen antioxidant) and outside the body which comes from plants (Thangavelu, *et al*., 2015). Research into plants with anticancer effects is actively encouraged in order to discover new drugs with lesser toxicity but more potent effects (Kaewthawee and Brimson, 2013; Elgadir, *et al*., 2015).

Indonesia has diverse plant species which can be utilized as medicinal plants. One of these medicinal plants is *Plectranthus amboinicus* (Lour.) Spreng. This plant was reported to contain ursolic acid which is a pentacyclic triterpenoid carboxylic acid and pharmacologically active (Shan, *et al*., 2009; Wang, *et al*., 2011). It is one of the chemopreventive agents which able to suppress the cancer cell proliferation and induce apoptosis (Gupta, *et al*., 2015; Kamuhabwa, *et al*., 2000). Previous studies have showed that the ethyl acetate extract with the highest amount of phenolic compounds exhibited the greatest antioxidant activity (Hasibuan, *et al*., 2013).

The *in vitro* cytotoxic property of the leaves crude extract was tested against cervical adenocarcinoma (HeLa) cells, and the result displayed cytotoxic effect of n-hexane, ethyl acetate and ethanol extracts on HeLa cells with IC_{50} values 76.322 µg/mL, 143.291 µg/mL, and 88.997 µg/mL, respectively (Rosidah and Hasibuan, 2014). It also showed cytotoxic effect on MCF7 breast cancer cell lines concurrently (Hasibuan, *et al*., 2013).

The previous studies showed that n-hexane, ethylacetate extracts exhibited strong cytotoxic effect on T47D breast cancer cells with IC_{50} value of 44.716 µg/mL and 37.61 µg/mL, respectively and showed the synergistic effect in combination with doxorubicin to inhibit the HeLa cell line (Hasibuan and Rosidah, 2016). It displayed the same effect in combination with doxorubicin to inhibit T47D cell line (Hasibuan, *et al*., 2015). Thus, the extract has potential effect as a chemoprevention. The aims of this study are to investigate the antioxidant and cytotoxic activities of the ethyl acetate fractions of *Plectranthus amboinicus* (Lour.) Spreng on HeLa and T47D cell lines.

MATERIALS AND METHODS

Chemicals and reagents

Ethyl acetate, n-hexane were purchased from Merck (Darmstadt, Germany), so does $AlCl_3$ and natrium acetate. Meanwhile 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and quercetin were from Sigma Chemical (St. Louis, USA). Silica gel 60H and dimethyl sulfoxide (DMSO) were from Sigma Aldrich Chemie GmbH (Schnelldorf, Germany). RPMI media and Phosphate Buffer Saline (FBS) 10% v/v were from Gibco (New York, USA).

Preparation of extract and fractions

The *Plectranthus amboinicus* was obtained from Pematang Siantar, North Sumatera, Indonesia. The leaves of *Plectranthus amboinicus* were dried at 45^{o}C for 7 days in drying cabinets and ground into powder, then followed by extracted with n-hexane through maceration method for three days at room temperature. The supernatant was separated by decantation and the marc was remacerated twice. The extracted marc was then re-extracted with marc of ethyl acetate by maceration. Extract from each solvent were concentrated by a rotary evaporator (Heidolph VV-200, Sigma Aldrich Chemie GmbH) and the concentrated extract was dried by freeze-dryer (Edwards, London, England). The extract was fractioned with n-hexane, and ethyl acetate with vacuum liquid chromatography by using gradient eluent (100:0; 90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100).

In stationary phase, ethyl acetate fractions was fractionated with gradient eluent ethyl acetate: methanol (100:0; 90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100), methanol (100) and silica gel 60H. All fractions were concentrated by rotary evaporator and were freeze-dried to

eliminate any remaining water. Then, the fractions were analyzed by thin layer chromatography with silica gel GF254 and selected n-hexane-ethyl acetate eluent.

Determination of DPPH scavenging activity

The free radical scavenging activity of *Plectranthus amboinicus* ethyl acetate fraction, and quercetin was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH (Rosidah, 2008; Atolani and Olantuji, 2016). Each of 7.5 mL; 8.75 mL; 10 mL; 11.25 mL from *Plectranthus amboinicus* extract and each of 0.25 mL; 0.5 mL; 0.75 mL from Quercetin (in methanol) were placed in different test tubes. To this mixture, 5 mL 0.5 mM DPPH was added. After 30 min of incubation at room temperature (22-24^{o}C), absorbance was measured at 517 nm by using spectrophotometer (Shimadzu, Kyoto, Japan) with methanol as the blank. A control contained 1 mL methanol and 5 ml 0.5 mM DPPH. Free radical scavenging activity of the extracts (%) was calculated according to the following formula: (Ac – As)/Ac x 100. Where As is the absorbance of DPPH and sample and Ac is the absorbance of control.

Total flavonoid content (TPC) method

Two mL of ethyl acetate fraction of *Plectranthus amboinicus* (Lour.) Spreng. was blended with 0.1 mL aluminium chloride ($AlCl_3$) 10% reagent (1:10) in the reaction tube. It was mixed and incubated at room temperature for 5 minutes. 0.1 mL natrium asetat (CH_3COONa) and 2.8 mL water was added into the solution, mixed and incubated at room temperature for 60 minutes. The absorption was measured by spectrophotometry UV-Vis at 752 nm. The flavonoid content of ethyl acetate fraction of *Plectranthus amboinicus* (Lour.) Spreng. was calculated by substituting the mean value of sample absorbance to regression equation which was obtained from the calibration curve. The total flavonoid content was presented in milligrams of quercetin per gram of sample (mg QE/g sample) (Rosidah, *et al.*, 2008; Asrin, *et al.*, 2017; Satria, *et al*., 2017)

Cell lines and culture conditions

HeLa (cervical adenocarcinoma), T47D (ductal breast adenocarcinoma) and MCF7 (pleural effusion breast adenocarcinoma) cell lines were provided by Paracitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The cell lines were cultured in RPMI (Gibco) for HeLa cells and DMEM (Merck) for T47D and MCF7 media, supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma Aldrich Chemie GmbH), 2% penicillin-streptomycin and 0.5% fungizone (Gibco) in a 37^{o}C incubator with 5% CO_2.

Cytotoxicity assay

Cytotoxicity was determined by MTT colorimetric assay. Briefly, HeLa, T47D and MCF7 breast cancer cell lines were plated at 10^4 cells/well in a 96-well plate. Each well contained $1x10^4$ cells, followed by incubation 24 h at 37^{o}C. Cells were treated with ethyl acetate extract of *Plectranthus amboinicus* (Lour.) Spreng. at various concentrations for 24 hours.

Cells were added with MTT 0.5 mg/mL solution and incubated for 4 hours at 37^{o}C. The reaction was stopped by adding stopper solution containing 10% SDS (Sigma Co, St. Louis, USA) in 0,01 N HCl (Merck, New Jersey, USA) and incubated overnight in room temperature. Absorbance was measured by a plate ELISA reader at λ 595 nm (Bio-rad, California, USA). Percentage of viable cells was calculated from the absorbance data. Percentage of viable cell = (Absample-Abmedium)/(Abcontrol-Abmedium) x 100%. Where A, B and C are absorbance of control group, treatment group and medium (vehicle), respectively (Hameed, *et al*., 2016; Illian, *et al*., 2018).

Statistical analysis

All data was stated in IC_{50} which was analyzed by using probit in regression at SPSS 19. The results were expressed as mean±SEM. The test was then

used for statistical analyses with *p*-values of 0.05 and were considered significant.

RESULTS

The result of fractioning test has identified by using thin layer chromatogram were presented in Table 1. These fractions that used as samples for antioxidant and cytotoxic activity test. The antioxidant activity of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. was identified through the color change of DPPH from purple to yellow; when odd electron from DPPH radicals paired with hydrogen from the extract. The result of 5 concentrations of each ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. with 5th times repeating can be seen on Figure 1.

The result of linear regression and IC_{50} value of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. can be seen on Table 2. A substance possesses antioxidant properties when the IC_{50} value is lower than 200 µg/mL (Molyneux, 2004). From Figure 1 showed that the fractions of ethyl acetate of Plectranthus amboinicus, (Lour.) Spreng. have a moderate DPPH scavenging activity.

Quercetin is used as a standard in order to determine the total flavonoid content. The absorbance value of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. was plotted against the quercetin curve with correlation coefficient value of 0.99665. The total flavonoid content is expressed in QE (quercetin equivalent), *i.e*., the amount of milligram quercetin equivalent in 1 gr of sample (Geetha, *et al*., 2017). The result of total flavonoid content is displayed on Figure 2.

Figure 2 exhibited that fraction III has the highest flavonoid content. Whereas, fraction II has highest IC_{50} value when compared with the result of DPPH assay. The total flavonoid contents in the fractions described the antioxidant containing in each fractions. TPC was determined by the Folin–Ciocalteau method (Cicco, 2009). The fraction II of *Plectranthus amboinicus* was found to contain low levels of phenolic content 50 mg GAE/g. Phenolic compounds are known as an antioxidant, and they are very important plant constituents because of their free radical scavenging ability due to their hydroxyl groups (Sun and Ho, 2005).

In this study, MTT test was conducted to evaluate the cytotoxic activity of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. on HeLa, T47D and MCF7 cell lines. The cancer cells were exposed to various concentrations (31.25 - 500 µg/mL) of ethyl acetate fractions for 24 h. As seen on Figure 3, the higher concentration

Table 1. Rf value of *Plectranthus amboinicus*, (Lour.) Spreng. Fraction by using thin layer chromatogram.

Fraction	Moving Phase	Rf value
I	n-hexane : ethyl acetat (100:0)	0.38; 0.4067; 0.5733; 0.667; 0.68
II	n-hexane : ethyl acetat (90:10; 80:20)	0.5733; 0.6667
III	n-hexane : ethyl acetat (70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90)	0.28; 0.8; 0.57; 0.637; 0.68
IV	n-hexane : ethyl acetat (0:100)	0.4; 0.5733; 0.7067
V	ethyl acetat : methanol (100:0; 90:10; 80:20; 70:30; 60:40; 50:50)	0.24; 0.4; 0.5067; 0.5733; 0.6267; 0.68

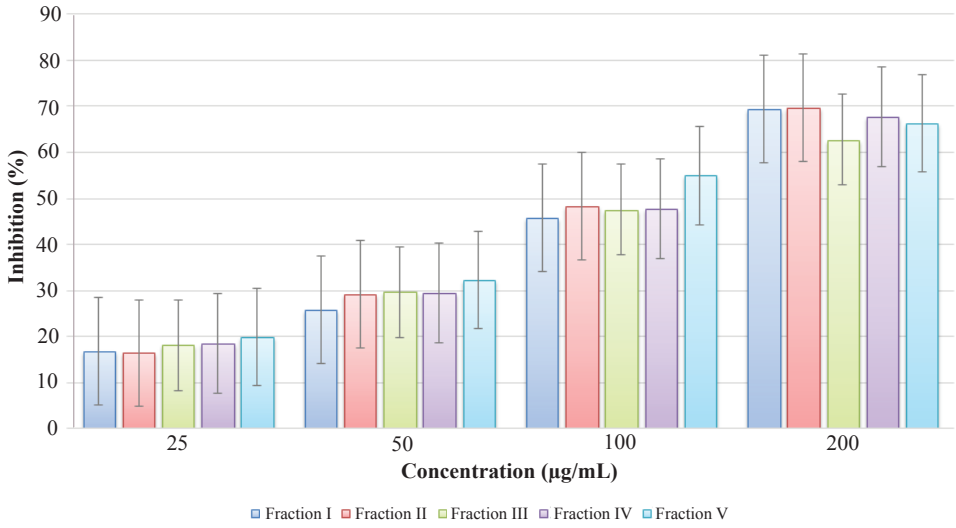

Figure 1. Antioxidant activity of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. by DPPH method.

of ethyl acetate fractions resulted in decreasing cell viability toward Hela cells. The cytotoxic effect of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. on T47D cells (Figure 4) showed the similar result to that of Hela cells (Figure 3).

The cytotoxic effect of *Plectranthus amboinicus*, (Lour.) Spreng. fractions on MCF7 cell lines have been shown in Figure 5. According to the results, a non-dependent concentration toxicity was observed in MCF7 cell for all fractions.

This result showed that the value of flavonoid content is not linear with the value of IC_{50}, because not all flavonoid compounds is capable of reducing free radicals which are observed from the differences in the structure of each type.

DISCUSSION

Phenolic compound play an important role in the prevention of cancer disease related to oxidative

Table 2. The antioxidant activity of ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. by DPPH method.

Samples	Regression equation	R value	IC_{50} (µg/mL)
Fraction I	Y = 0.333x + 6.617	0.96	130
Fraction II	Y = 0.335x + 7.632	0.95	127
Fraction III	Y = 0.295x + 9.485	0.91	137
Fraction IV	Y = 0.320x + 8.700	0.94	129
Fraction V	Y = 0.314x + 11.077	0.88	124
Quercetin	Y = 9.055x + 5.128	0.98	4.95

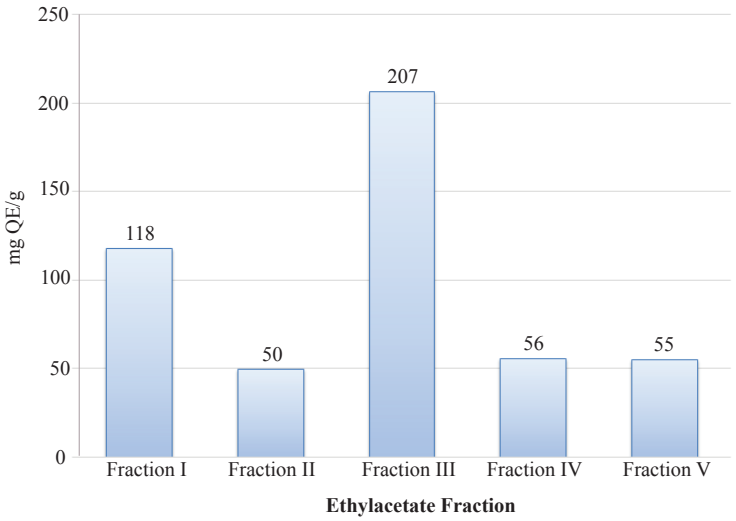

Figure 2. The Flavonoid Content of ethyl acetate fractions of *Plectranthus amboinicus* (Lour.) Spreng. by TPC method.

damage due to their antioxidant properties (Hameed, *et al*., 2016). The antioxidant activity of phenolic compounds depends on the structure, in particular the number and the positions of the hydroxyl groups and the nature of substitution on the aromatic rings (Middleton, *et al*., 2000)

Several studies of antioxidant activity show the difference in solvent fractions is closely related to the total content of phenolic present in them. It is also known that antioxidant activity of a plant extract is not only limited to phenolic and flavonoid but also another compounds, depend on the solvent extraction (Sun and Ho, 2005; Choirunnisa, *et al*, 2016). Therefore, the relationship between total flavonoid levels and antioxidant activity among plant extracts is complex (Pokorny, *et al*, 2001).

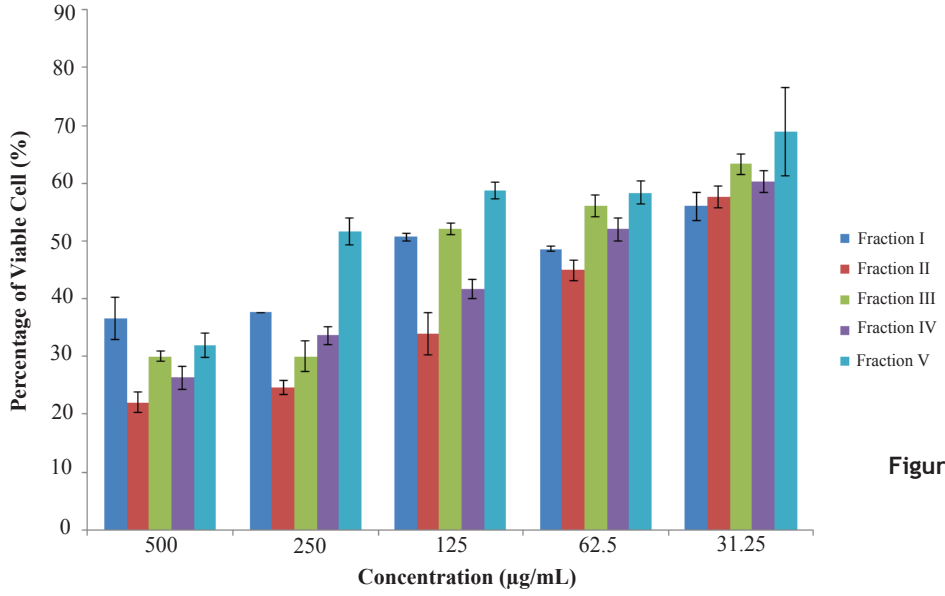

Figure 3. Cytotoxic effect of ethyl acetate fraction of *Plectranthus amboinicus*, (Lour.) Spreng. on HeLa cell line.

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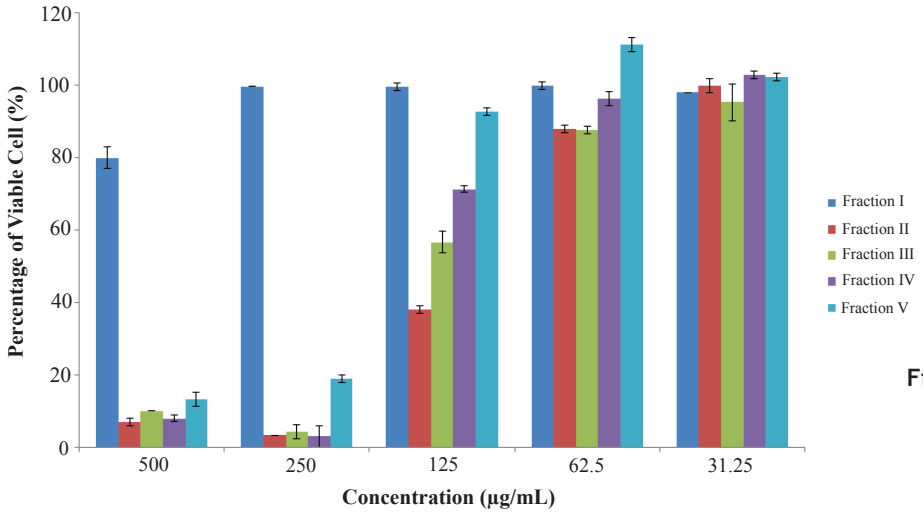

Figure 4. Cytotoxic effect of ethyl acetate fraction of *Plectranthus amboinicus*, (Lour.) Spreng. on T47D cell line.

The cytotoxic activity evaluated by the MTT assays with corresponding IC_{50} are summarized in Table 3. The results showed that the ethyl acetate fractions of *Plectranthus amboinicus*, (Lour.) Spreng. had potent cytotoxic activities. The ethyl acetate fractions performed more potent cytotoxic activities on Hela than T47D and MCF7 cell lines. Fraction II exhibited significant cytotoxic activity on HeLa, followed by fraction IV, fraction I, and fraction III.

The fractions effect both on T47D and MCF7 cell lines showed unsatisfactory results. The lower IC_{50} values represent the higher potency of the extracts to inhibit the growth of cells. In order to be considered as a potential drug candidate, the IC_{50} value of such agent should be sufficiently low to avoid any possible unspecified effects (Anlar, *et al*., 2016).

The American National Cancer Institute assigns a significant cytotoxic effect of promising

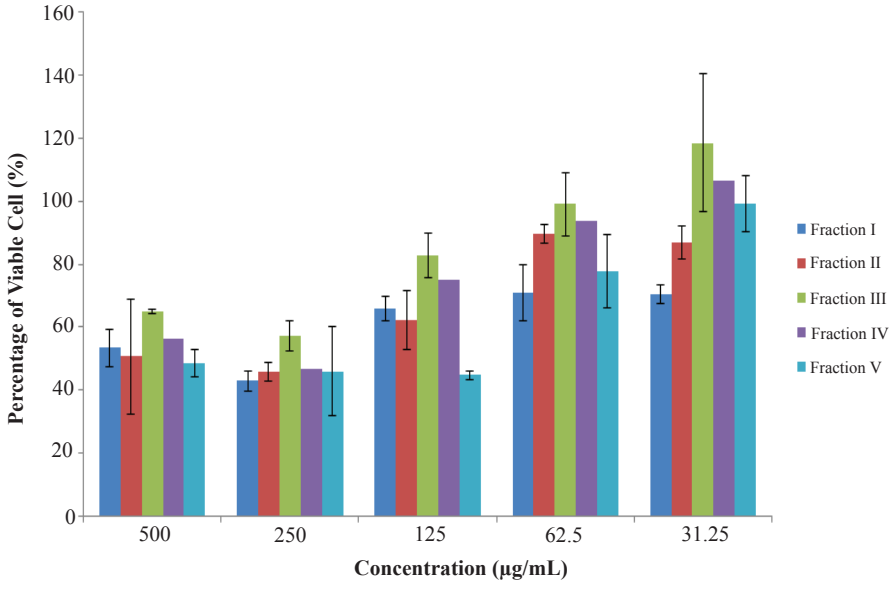

Figure 5. Cytotoxic effect of ethyl acetate fraction of *Plectranthus amboinicus*, (Lour.) Spreng. on MCF7 cell line.

Table 3. Cytotoxic activity (IC_{50}) of ethyl acetate fractions of *Plectranthus amboinicus* (Lour.) Spreng. on HeLa, T47D and MCF7 cell lines.

Fractions	HeLa (µg/mL)	T47D (µg/mL)	MCF7 (µg/mL)
I	77.076	1621.37	259.71
II	46.045	111.19	343.74
III	93.169	127.68	575.11
IV	71.439	149.54	408.54
V	476.009	208.5	250.57

anticancer product for future bio guided studies, which the IC_{50} value should exert under 30 µg/mL (Hasibuan, *et al.*, 2013). However, according to Kamuhabwa (2000), the potential cytotoxic activity of the extract is less than 100 µg/mL. In this study, the focus is on ethyl acetate fractions, whereas the cytotoxic activity could be due to the presence of active compounds like flavonoid that could probably have inhibitory effects on the cancer cell lines. Several studies have shown high cytotoxic and anticancer activities of flavonoids (Mahadev, *et al*., 2015). The recent study showed that ethylacetate fraction of Picria fel-terrae could inhibit cell grow on G0/G1 phase and induced apoptosis (Satria, *et al*., 2017). Ethylacetate contained flavonoid which were capable to scavenge the reactive oxygen species effectively because of the phenolic hydroxyl groups and so they are potent antioxidant. Flavonoids reduce breast cancer cell proliferation by inhibiting cell growth, protein kinase activities, and induction of apoptosis (Wang, *et al*., 2011; Sun, and Ho, 2005).

CONCLUSION

It can be concluded that *Plectranthus amboinicus*, (Lour.) Spreng. ethyl acetate fractions showed DPPH scavenging activity in medium category with the lowest IC_{50} is 124 µg/mL in fraction V and the highest is 137 ppm in fraction III. The lowest total flavonoid content 50 mg QE/g in fraction II and the highest 207 mg QE/g in fraction III. Fraction II from ethylacetate extract of *Plectranthus amboinicus*, (Lour.) Spreng. exhibited highest cytotoxic effect both on HeLa and T47D but not on MCF7 cell lines. Fraction V is the most cytotoxic on MCF7 cell line. Required further study to isolate the active compound.

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