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CYTOTOXIC ACTIVITY AND APOPTOTIC INDUCTION OF LEAVES AND FRUIT EXTRACT of SCREW PINE (*Pandanus tectorius*) TO T47D CELL LINE

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Abstract

Breast cancer is one of cancer types that can lead to human death. Beside that, Indonesia has a lot of potential anticancer herbs, among them is from genera *Pandanus*. Anticancer potency can be determined by cytotoxic activity on the cancer cells. A species of genera *Pandanus* that can be found in Indonesia is *Pandanus tectorius*. *Pandanus tectorius* is abundant in the area of Gunungkidul, D.I. Yogyakarta but its anticancer potency is not explored yet. So, its important to explore anticancer potency that possible on *Pandanus tectorius*. This research aimed to study the cytotoxic and apoptosis induction activity of the leaf and fruit extracts of *Pandanus tectorius* on breast cancer cells (T47D). The plant materials were sampled in Gunungkidul, D.I. Yogyakarta. Extraction was done by maseration method using ethanol, chloroform, and aquadest independently. The extracts were subjected to Thin Layer Chromatography for identifying compound groups; cytotoxic activity was assessed by the MTT assay in triplicate. Statistical analysis was conducted by ANOVA, LSD test with Tukey HSD and probit analysis to determine the IC_{50}. Extracts with the lowest IC_{50} value were subjected to double staining method (Ethidium-Bromide Acrydine-Orange) to differentiate necrotic from apoptotic death pathway of cell death. The results showed that IC_{50} values from low to high were aquadest leaf extract (61.28 µg/mL), ethanolic leaf extract (105.09 µg/mL), chloroform leaf extract (222.76 µg/mL), and ethanolic fruit extract (773.69 µg/mL), while the IC50 values of chloroform fruit extract and aquadest fruit extract could not be determined. Aquadest and ethanolic leaf extracts showed a low apoptosis induction ability, suggesting that the cell death was through non-apoptosis pathway tautophagy pathway. It might be concluded that aquadest and ethanolic leaf extracts has potency on cytotoxic activity towards breast cancer cell in T47D cell culture.

Keywords: Cytotoxic activity, Apoptosis induction, T47D cell line, *Pandanus tectorius*

INTRODUCTION

Cancer is a complex disease that can cause death to the sufferer. One of cancers that causes a high rate of death is breast cancer. Breast cancer is the second killer in the world and sixth in Indonesia [1]. Commonly, death happens because of late diagnosis and treatment to the sufferer. Breast cancer not only attacks women, but also men. So far, the treatment to cure breast cancer include surgery, radiation therapy, hormonal therapy, gene therapy, and chemotherapy. However, such therapy is not completely safe because chemotherapeutic agents do not only attack cancer cells but also normal cells such as hair cells, bone marrow and gastrointestinal cells. In addition, the chemotherapy drug category 1 can also cause nausea and vomiting in 70-80% of chemotherapy patients [2].

Based on previous research that was conducted [3,4], *Pandanus conoideus* fruit on red and yellow varieties from Papua are promising as sources of bioactive compounds including anticancer agents. However, this plant is only found in Papua. Urging the search for more available, abundant, inexpensive, and safe of natural resources of anti-cancer agents.

In the other hand, Indonesia has many potential anticancer herbs, one of them is *Pandanus tectorius* that is abundance in Gunungkidul, D.I. Yogyakarta. Gunung kidul area has the most abundant of that plants among other districts in the province, which is 211.2 hectares. However, the uses of this plant have not been optimal. The production of *Pandanus tectorius* in Gunungkidul is the lowest (10,84 tonnes/hectare) among other districts [5]. Utilization is still limited to the use of the leaves as handicrafts such as woven bags, a variety of baskets, mats, chairs, and tables, while the fruit is unused and unutilized.

Chemical compound of plants in the same genera is possible to have similar content and function. So, *Pandanus tectorius* might have a potential as anticancer agents. In addition, anticancer assay on the leaves and fruit of *Pandanus tectorius* has not been done. In this study, the anticancer potential of *Pandanus tectorius* extracts was assesed by cytotoxicity and apoptosis induction assay as preliminary and screening test that is cheaper, easier, and

ISSN:2337-442X

more accurate. This research wasaimed to study the cytotoxic and apoptosis induction activity of the extracta of the leaf and fruit of *Pandanus tectorius* on T47D cells (breast cancer cells).

MATERIALS AND METHODS

Plant Material and extraction

Fruit was collected in mature conditions that indicate from the orange colour of exoderm. Then leaves were sampled based on uniformity of size and colour. Leaves and fruit were collected from Kukup Beach, Gunungkidul, and D.I.Yogyakarta. Fruit mesoderm was separated from exoderm and it seeds and cut it small. Leaves and fruit were dried on the 40^{o}C and after dry, both leaves and fruit were grinded to powder. By maseration method, leaves and fruit powder were extracted by ethanol 96%, chloroform and aquadest independently.

Secondary metabolite detection by Thin Layer Chromatography (TLC)

Leaves and fruit extract were fractionated by Thin Layer Chromatography (TLC) and detected secondary metabolite alkaloid, flavonoid and tannin. It used solvent such as n-hexane, ethanol 96%, and chloroform independently. Extract spotted in the silica plate 254 and put inside TLC glass using eluen Methanol: Ammonia 100:1,5 (Alkaloid), Butanol : Acetic acid : Aquadest 3:1:1 (Flavonoid) and Etil acetic : Formiac acid : Acetic acid : aquadest 100:11:11:27 (Tannin). Then it observed below 254 and 365 nm wavelength UV light and sprayed by Citroborate (Alkaloid), Dragendorf (Flavonoid), and $FeCl_3$ (Tannin). Racing factor (Rf) can be calculated by appeared spot in silica plate using formula below:

$$\text{Rf value} = \frac{\text{Distance center of spot to the begin spot}}{\text{Distance of eluen}} \qquad [6]$$

Cytotoxic Assay by MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) [7]

Cytotoxic activity measured by MTT assay. T47D cell line is collected from ***Integrated Research and Testing Laboratory*** (LPPT) UGM. T47D cells cultured in complex medium (87,5% RPMI+10% FBS+0,5% Fungizon+0,5% Penstrep) inside Flask. After confluent, its ready to cells harvest and count by haemocytometer. Each well on 96 platewell is filled by 2 x 10^4cells/100 µL/well on complete medium. 96 platewell incubated at 37^{o}C, CO_2 5% for 4 hours. Treatment is performed in triplicate. There were 4 variable that assessed, consist of cells control (T47D cells suspensionin complete medium), Solvent control (T47D cells suspension + complete medium + DMSO 0,125%; 0,25%; 0,5%), Treatment group (Each extract by consentration7,815; 15,625; 31,25; 62,5; 125; 250; 500 and 1.000 µg/mL on each 100µL/well) and positive control (Doxorubicin at consentration 3,906; 7,815; 15,625; and 31,25 µg/mL). After treat the cells, 96 plate well incubated on 37^{o}C, CO_2 5% during 24 hours. In the end of incubation, every well added 10 µL MTT (5 mg/mL) and incubated on 37^{o}C, CO_2 5 % during 4 hours. Stopper reagent, SDS 10% in HCl 0,1 N as much as 100 µL/well, stop MTT reaction. Then, Platewell assessed by *ELISA reader* (550 nm). Inhibition percentage calculated by below formula:

$$\% \text{ Inhibition} = \frac{\text{(Cells control abs. – treatment abs.)}}{\text{Cells control abs.}} \times 100\% \qquad [7]$$

Then, probit analysis calculated to know IC_{50} (Inhibited Concentration 50) and one-way ANOVA analysis, LSD by HSD Tukey.

Apoptosis Assay by Double Staining Method [8]

The potential extracts with lowest IC_{50} were selected and assessed for apoptotic induction on T47D cell line using Double Staining Method (Ethydium-Bromide Acrydine-Orange). T47D cell line was collected from **Integrated Research and Testing Laboratory** (LPPT) UGM and cultured in complex medium (87,5% RPMI+10% FBS+0,5% Fungizon+0,5% Penstrep) inside Flask. After cell has become confluent, it's ready to harvested and counted by

ISSN:2337-442X

haemocytometer. Then, 24 platewells were filled by coverslips and 5 x 10^4cells/100 µL/well on complex medium. Group variables of this research were cells control (T47D cells suspension in complex medium), solvent control (T47D cells suspension + complex medium + DMSO 62,5 and 125 µg/mL), Treatment group (Each extract by consentration 7,815; 15,625; 31,25; 62,5; and 125 µg/mL each 100 µL/well) and positive control (Doxorubicin by consentration 7,815 and 15,625 µg/mL). After treatment, 24 platewells incubated on 37^{o}C, CO_2 5% for 24 hours. After incubation, medium removed from wells and washed by PBS. Then coverslip moved to the object glass and added by 20 µL of EthydiumBromide+Acrydine Orange. Cell observed and counted by fluorescence microscope. Viable cells determined by green coloured cells and red coloured cells for death cell. Then, apoptotic percentage was calculated.

RESULT AND DISCUSSION

Secondary metabolite in leaf and fruit extracts

The result (table 1) showed that leaf and fruit extracts have a different Rf value. That mean it has a different secondary metabolite compound too. Tannin were not detected on leaf and fruit extracts. Alkaloid was detected only on leaves extract. Alkaloid has a function as medicine because it high biological activity. In low consentration, alkaloid can become therapeutic agents [9,10]. It means, these alkaloid function is unable in fruit extract . Overall, alkaloid, flavonoid and tannin detected on leaf and fruit extracts.

Table 1.Rf values of secondary metabolite compound

Secondary Metabolite compound	n-hexane extract		Ethanolic extract		Chloroform Extract	
	Leaves	Fruit	Leaves	Fruit	Leaves	Fruit
Alkaloid	0,42 ; 0,82	ND	ND	ND	ND	ND
Flavonoid	-	-	0,84	0,89	0,53	0,88
Tanin	-	-	ND	ND	ND	ND

Abbreviations: ND, 'Not determined'

Cytotoxic activity of leaf and fruit extracts

The inhibition percentage (%) of treatment groups of *Pandanus tectorius* extracts, solvent control (DMSO), cells control, medium control and positive control (Doxorubicin) showed in Figure 1. Based on the inhibition percentage of each treatment, IC_{50} value was determined (Table 2). Cytotoxic activity was conducted by MTT assay method. Using of various extracts solvent aimed to understand of polarityvariance in the compound that found in fruits and leaves of *Pandanustectorius* and understand the compound that more potent as an anticancer agents. Solvent controls used to ensure that the DMSO is not toxic to the cells so that the cytotoxic effect that happens is the effect of the extracts and doxorubicin. Based on statistical analysis using ANOVA, it is known that the value of inhibition percentage solvent control and cells control is not significantly different so it can understand that DMSO is not toxic and does not affect T47D cells viability.

Probit analysis can be performed to determine the IC_{50} values. IC_{50} (Inhibition Concentration 50) is a concentration that can kill 50% of cells in cell populations and determine the potential value of a compound in inhibiting cells growth. The lower values of IC_{50} determine higher potency of chemical compound to inhibit cell growth. It also has lower extracts concentration to kill cells by the same amount. Results shows that chloroform fruit extract and aquadest fruit extract have a fluctuation inhibition percentage so the probit analysis and IC_{50} values cannot to determine. IC_{50} values from low to high is aquadest leaf extracts (61.28 µg / mL), ethanolic leaf extracts (105.09 µg / mL), chloroform leaf extracta (222.76 µg / mL), and ethanolic fruit extracts (773, 69 µg / mL) (Table 3). Based on these results, it appeared that the whole leaves extracts had IC_{50} values whereas the fruit extract found only in ethanolic fruit extract. Its mean, compounds that potential as anticancer have a wide distribution from polar to non-polar. The most potent extract was aquadest leaf extracts (61.28 µg / mL).

ISSN:2337-442X

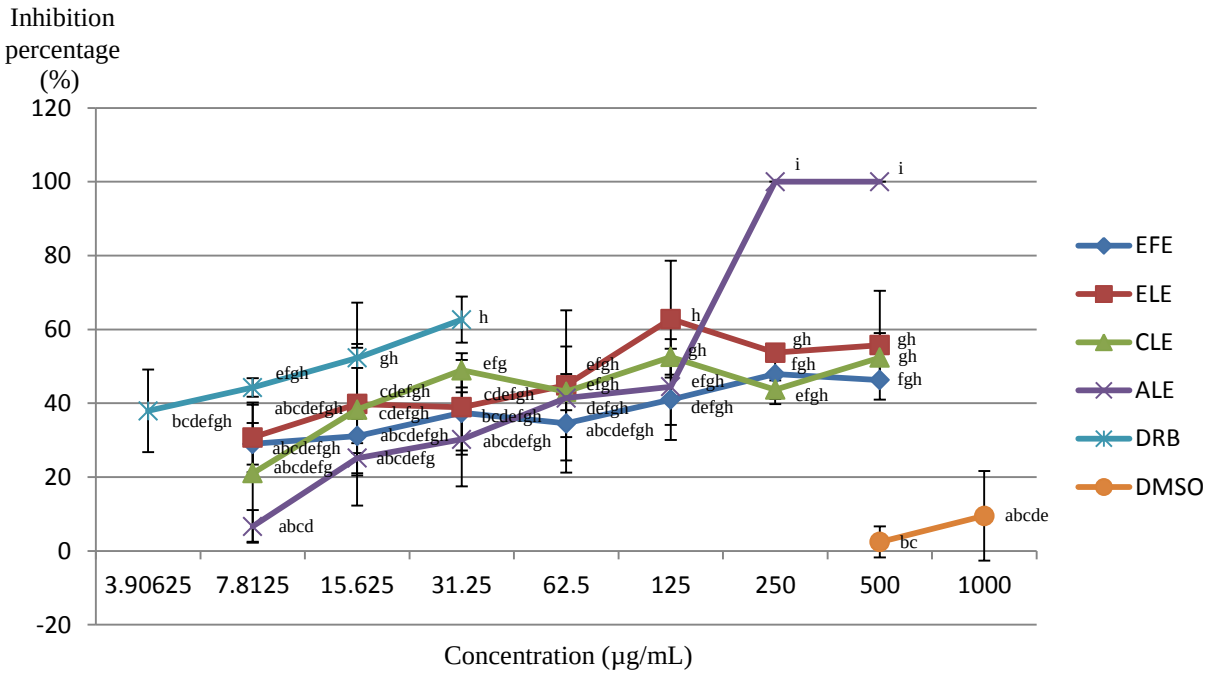

Figure 1.Inhibiton Percentage of leaf and fruit extracts to T47D cells

Abbreviation : ELE, Ethanolic leaf extract; CLE, Chloroform leaf extract; ALE, Aquadest leaf extract; EFE, Ethanolic fruit extract; CFE, Chloroform fruit extract; AFE, Aquadest fruit extract; DRB, Doxorubicin; ND, 'Not determined'.

Note: different letters in the same point indicates no significant difference (p >0.05)

Leaf extract showed higher cytotoxic activity. It possible that secondary metabolites such as alkaloids and flavonoids were detected in the leaf while alkaloids were only detected on leaf extracts. Then, leaf gets more intensively environmental stress compare to fruit. Environmental stress can trigger the production of secondary metabolites. Later studies on *Pandanus odoratissimum* leaf extracts showed that antioxidant activity, the ability to reduce predation and free radicals activity that can inhibit DNA to lead cancer [11]. The presence of antioxidant activity also indicates a higher cytotoxic activity. *Pandanus tectorius* fruit's have a physical protection against stress such as thick and hard of exocarpium. It make possible that the production of secondary metabolites in the fruit will be lower than in the leaf. In addition, the fruit has a major role as a food storing of the primary metabolite.

Table 2. IC_{50} of leaf and fruit extracts to T47D cells

	Ekstrak						
	ELE	CLE	ALE	EFE	CFE	AFE	DRB
IC_{50} (µg/mL)	105,09	222,76	61,28	773,69	Nd	Nd	11,74

Abbreviation : ELE, Ethanolic leaf extract; CLE, Chloroform leaf extract; ALE, Aquadest leaf extract; EFE, Ethanolic fruit extract; CFE, Chloroform fruit extract; AFE, Aquadest fruit extract; DRB, Doxorubicin; ND, 'Not determined'.

Leaf extract showed higher cytotoxic activity than fruit extract because it have more secondary metabolite detected, such as alkaloid and flavonoid. In spite of, leaf gets more intensive environmental stress than fruit so it can trigger secondary metabolite producing. Previous research showed that leaf extract of *Pandanus odoratissimum* has antioxidant activity and free radical reducing and scavenging. It can inhibit DNA damage that can trigger cancer [11]. Antioxidant activity possibly to determine that it has higher cytotoxic activity.

ISSN:2337-442X

The cytotoxic mechanism of *Pandanus tectorius* possibly through the phenol-flavonoids pathway because of the 10 phenolic compounds and 5 flavonoids were detected on *Pandanus tectorius* fruit extract (Zhang, 2012). In addition, some species from genera *Pandanus* has bioactivity were also associated with the presence of phenols and flavonoids. The different fraction of *Pandanus conoideus* extracts have antioxidant activity with IC_{50} 5, 25 to 53, 47 µg/mLthat has a positive correlation with the total phenols (r^2 = 0.645) and flavonoids (r^2 = 0.709) [13]. *Pandanus fascicularis* also has significant antioxidant activity [14]. *Pandanus odoratissimum* root extract have antioxidant activity and positive correlation with the content of phenol and flavonoid [15], as well as aquadest and methanol extracts of *Pandanus foetidus* that has antioxidant activity too [16]. It also showed that the cytotoxic activity will increase as well as phenol and flavonoid content increase.

Previous research showed that the petroleum ether leaves extract of *Pandanus raodorus* was non-polar compounds to generate lower IC_{50} [17]. In contrast to the results of this study, the polar aquadest extracts have the lower IC_{50}. It showed that polarity of natural compounds of both extracts is different despite come from the same genera. Therefore, to determine the specific compounds, such as the research conducted before [7]. In that study, it was found that the fraction of leaves extract has more potential anticancer compound, ie the fraction of the second and fourth fractions.

The result (Table 1.) showed that aquadest leaf extract has the lowest IC_{50} value than all treatment. It is suspected that the anticancer compounds were polar. The doxorubicin had lower IC_{50} than aquadest leaf extracts. It shows that doxorubicin was more toxic than extracts used. However, there are extracts with relatively low IC_{50} values is ethanolic leaf extract (105.09 mg / mL) and aquadest leaf extract (61.28 mg / mL) will be tested ability to induce apoptosis. It possible that both of ethanolic and aquadest leaf extracts also have apoptosis induction ability.

Apoptosis induction ability of potential extract

The result (figure 2) showed that the treatment with a higher apoptosis percentage is ethanolic leaf extract 7,81µg/mL (10,25%). But, higher concentration of ethanolic leaves extract gives an unstable apoptosis percentage. As well as on aquadest leaves extract have an unstable apoptosis percentage. On the doxorubicin treatment to the cell, all of the cells are dead and disappear from the cover slip because it had a high competence to induce apoptosis. On a DMSO treatment, it showed that apoptosis percentage is too low so it has no ability to induce apoptosis.

Apoptosis percentage should have a similar value with inhibition percentage on cytotoxic assay. But, on this assessment, showed that apoptosis percentage totally disimiliar with inhibition percentage. It possible the cell death pathway was not throught apoptosis pathway. We couldn't prove the evidence of apoptosis as the reason of cellular death in this study. Further evidence of apoptosis process can detect by electron microscopy, TUNEL staining, annexin staining, caspase-activity assays, detection of increased number of cells in subG1/G0 and detection on changes in mithocondrial membrane potential. But, the possibility for cell death pathway is throught lysosome-dependent pathway, such as autophagy or necrosis pathway because of the membrane cell degradation characteristic similar with apoptosis pathway. This caspase-independent pathway can lead cell degradation by lysosomal processes [18]. This lysosome-dependent pathway is important to maintenance of large protein and entire organelle such as mitochondria. Beside that, abnormalities of autophagy function are related to neurogedereration, heart disease and cancer [19, 20]

Apoptosis percentage
(%)

ISSN:2337-442X

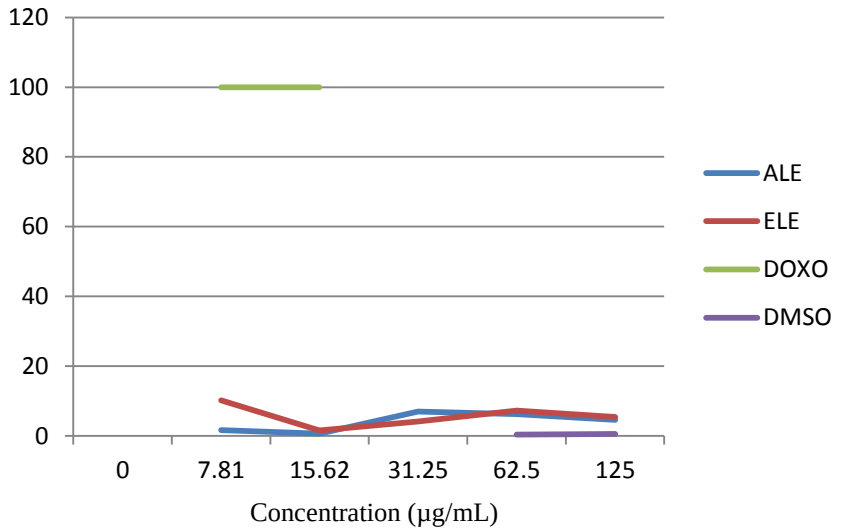

Figure 2. Apoptosis percentage of Aquadest leaves extract (ALE), Ethanolic leaves extract (ELE), Doxorubicin and DMSO to T47D cells

IC_{50} value of aquadest leaves extract (61,28 µg/mL) showed have the potential antiproliferation. According previous research, if the extracts had IC50 values ≤ 100 mg / ml can be determine to have the potential antiproliferation [21]. But, MTT assay based NCI (National Cancer Institut) criteria showed that a compound have cytotoxicity effect if it has LC_{50} values ≤ 20 µg/mL. On the other hand, established standards for natural ingredients that can be developed as anticancer is ≤ 50 µg/ml [22]. So, IC_{50} values of aquadest leaf extract (61.28 µg/mL), ethanolic leaf extract (105.09 µg/mL), chloroform leaf extract (222.76 µg/mL), and ethanolic fruit extract (773.69 µg/mL) were not significantly inhibit cell proliferation in T47D. It needs further research by fractionation and isolation of bioactive materials on the extracts and assess against the other cancer cells.

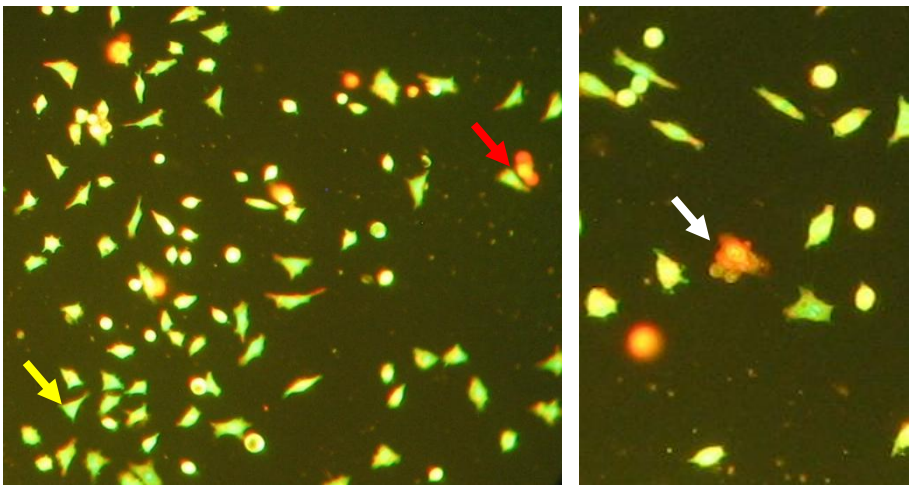

Figure 2. T47D cells after treatment of leaf extracts of *Pandanus tectorius* stained by Ethydium-Bromide Acrydine-Orange. T47D cell undergo apoptosis (red arrow), viable cell (yellow arrow) and blebbing cell (white arrow).

CONCLUSION

ISSN:2337-442X

Alkaloid was detected only on leaves extract and flavonoid detected on both leaves and fruit extract of *Pandanus tectorius*. Leaves and fruit extract of *Pandanus tectorius* have a cytotoxic activity to T47D cell, especially on ethanolic leaf extract, chloroform leaf extract, aquadest leaf extract and ethanolic fruit extract. IC_{50} value of aquadest and ethanolic leaf extract are more potential as anticancer agents. We couldn't prove the evidence of apoptosis as the reason of cellular death in this study. Cell death pathway was possible throught non-apoptosis pathway.

ACKNOWLEDGEMENT

We acknowledge to Student-Lecture Collaboration Grant of LPPM UGM as financial funding, Laboratory of Assessment and Integrated Services (LPPT-UGM) and Ms. Woro Anindito Sri Tunjung on Laboratory of Biochemistry Faculty of Biology UGM.

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ISSN:2337-442X