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The Effect of Crude Extract of *Pandanus conoideus* Lamb. var. Yellow Fruit on Apoptotic Expression of the Breast Cancer Cell Line (T47D)

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ABSTRACT

A mechanism controlling a growing cancer cells is by a programmed cell death (apoptosis). The wildtype-*p53* enable to stop cleaves that follow DNA repair or cell death (apoptosis). The mutation of wt-*p53* caused loosing its ability to inhibit cancer cells proliferation. Healing methods like surgery, radiation, immunotherapy and chemotherapy still have some weaknesses, and clinical medicine to cancer is also still has any dissatisfactory. Much of chemotherapy was not given optimal result yet, because no specific action to cancer cells only, but also to the normal cells. These problems encourage important effort to find specific and sensitive anticancer. Empirical evidence indicates that the crude extract of *Pandanus conoideus* Lamb var. yellow fruit has potential effect as an anticancer. Method of Freshney was used in growing T47D cell line, counting cells was done by direct counting, and apoptotic evaluation was done by TUNEL enzymatic labeling assay. The results of the research demonstrated that the LC50 of yellow fruit extract are 0.25 µL/mL. The percentage of apoptotic of 0.125 µL/mL, 0.0625 µL/mL, and 0.03125 µL/mL are 34.38±2.26, 30.03±3.87 and 21.07±1.14 respectively.

Key words: T47D, Apoptotic, *p53*, *Pandanus conoideus* Lamb. var. yellow fruit.

INTRODUCTION

There are more than 30 kinds of *Pandanus* sp. (Widiyanto, 2006). Fourteen taxon from Pandanaceae were classified into red fruit, and wellknown for its beneficial use, while the rest of them belong to the group of yellow fruit, namely awone mengkaki by local people of Serui. *Pandanus conoideus* Lamb. at present is known by local people in that area as the red fruit, and this plant endemically growing in Papua. Four varieties of *P. conoideus* are cultivated by peoples in that area because its economical value as medicinal plant. Those are *P. conoideus* var. long red fruit, short red fruit, brown fruit, and yellow fruit. The importance of those fruits as traditional medicine provide prominent source as of new anticancer agent originated from Indonesia. The red fruit plant at present undergo over-exploitation, and its population reduced drastically due to utilisation of its fruits by traditional medicine producers both from Indonesia and other country. Empirical experience shows that *P. conoideus* var. yellow fruit (later abbreviated as yellow fruit) could act as anticancer, however, the mechanism underlying the inhibition of cancer cell by this fruit is still not fully understood yet.

The yellow fruit has been analysed by I Made Budi the person who found the red fruit, it contain tocopherol and β-carotene higher than that of red fruit (personal communication, 2007). Natural subtances found in this fruit such as carotene (9,500 ppm), β-carotene (240 ppm), tocopherol (10,400 ppm), and also oleic acid, linoleic acid and decanoic acid of omega 3 and omega 9 are known as powerfull antioxidant, aiding in preventing many deseases including cancer.

Breast cancer is the most common cause of death from cancer among women in the world. Mostly the victim of breast cancer (60-70%) was because of too late in testing it, so that it causes their death (Klauber-DeMore et al*.,* 2001). It globally takes the second place of women death after cervic cancer. In Indonesia, victim of breast cancer gradually increases by year, whereas United State reports that 27 in 100.000 (18%) of death are caused by breast cancer (Tjindarbumi and Mangunkusumo, 2002; Meiyanto et al*.,* 2006).

The main problem of chemoterapi is in it's low selection in anti-cancer medicine (Valeriote et al*.,* 2002; Kinghom et al*.,* 2003; Jenie and Meiyanto 2007). The use of radiation for theraphy like chemotheraphy and hormonal therapy, could result in

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any other effects to human body such as hairfall, skin getting darker (Jiang et al*.,* 2004). This problem has lead to promote the use of traditional medicine, which is generally believed to have less side effect (Sugiyanto et al*.,* 2003). One of the strategy to find the compound that works as target of its action to several gene which regulate the growth or proliferation of the cells (Gibbs, 2000).

The T47D breast cancer cell line (from ATCC, American Tissue and Culture Collection) is cell taken from epitelium of mamae ductus cell suffering malignation. This cell has a gene mutation of *p53* in the positive of amino acid 194th, with the fenilalanin amino acid (M;194F) (Nigro et al*.,* 1989). The mutation at the *p53* gene often found following a non regulated genetic as long as carsinogenesis in mostly tumor kind, included the breast cancer and cancer-derived cell lines (Smardova et al*.,* 2005). In this cells *p53* mutation occurred at the 194 residue (in the zinc binding domain L2), so that *p53* loose its function. If the *p53* is not link with DNA, so that the potential of regulating cell cycle and apoptosis could be reduce or completely lost (Schafer et al*.,* 2000).

The main principle of the effectifity and potential selection of anti cancer could be dealed with mutated *p53*, so that the apoptosis of cancer cells could proceeded. In general, the objective of the reseach were to promote the use of the variety of plantation in Indonesia, by using the yellow fruit especially as an agent which is cytotoxic, and provide scientific reasons of the use of the yellow fruit as a cancer medicine in society. The research on the cytotoxicity effect of yellow fruit extract toward cell line of cancer T47D should be investigated by observing the effect of cytotoxicity in inhibition mechanism of the cell growth (cycle of the cell) and apoptosis especially in relation with the expression of the *p53* gene.

MATERIALS AND METHODS

The T47D growth was monitored according to the method described elsewhere (Freshney, 2000). The cells linkage was counted using 20 µL of cell suspesion, added with 180 µL of tryphan blue, and the cells was then counted with haemocytometer at the fase contrast microscope. The total of cells found then multiplied with liquidity factor and number 10^4/mL (Fresney, 1987). The extract of the yellow fruit was taken from fresh yellow fruit. Solution of the test made by dissolving DMSO (dimethyl sulfoxide) 0.25% filtered with microfilter with diameter of 0.22 µm until the suspencion is in homogenious condition and then put in a sterile cup as main solution. Cytotoxicity test was done by pouring in 100 µL complete medium consist of cell suspension with closeness around $2x10^5$ cells/mL into each well 96 hole micro culture.

The effect of inhibited kinetic proliferation after treatment with yellow fruit toward cell T47D, inhibition test of proliferation kinetics was done for 7 hours, by counting the growth of cells (12, 24, 48, 72).

Apoptosis observation was done with TUNEL enzymatic labelling assay. Cell suspension was dropped to the slides and was incubated in poly-L lysine, fixed with 4% formaldehide in the PBS prior to permeabilization by Triton X-100. This step was followed by washing and rewashing using PBS. DNA was labelled with fluorescence-12-dUTP after TdT enzyme treatment. Slides were then covered with plastic coverslip and incubated at 37^{o}C for 1 hour, by avoiding direct exposure to sunlight/roomlight. To stop the reaction, coverslip was removed and SSC (Sodiun Saline Citrate) was added for 2x5 minutes. Slide were washed with PBS before addition of propidium iodine, then rewashed with PBS. Sample were analized under fluorescence microscope. The apoptotic cell will appear in green, while non-apoptotic in red. The treatment to control the positive TUNEL was done by adding DNA-ase I enzyme after permeabilization with TritonX-100 and beeing washed with PBS.

RESULTS AND DISCUSSION

The results given in Table 1. shows that the extract of yellow fruit inhibits the growth/proliferation of T47D cell. This experiment was performed to know the cytotoxicity potential of the yellow fruit extract toward T47D cell with parameter of LC_{50} percentage of T47D cell.

Table 1. The percentage of T47D cell death after treatment with the crude extract of *P. conoideus* var. yellow fruit

Concentration (µL/L)	The percentage of death			Mean ±SD
	I	II	III	
Control	0	0	0	0±0
4	100	100	100	100±0
2	92.68	95	92.68	93.45±1.34
1	72.09	68.18	70.45	70.24±1.96
0.5	59.57	58.33	59.57	59.16±0.72
0.25	50	50	50	50±0
0.125	40.38	39.62	48.83	42.94±5.11
0.0625	32.14	31.58	31.58	31.77±0.32
0.03125	26.66	26.23	26.23	26.37±0.25
0.0015625	20.63	19.05	19.05	19.58±0.91
0.0078125	13.64	13.64	13.43	13.57±0.12
0.00390625	5.7	7.04	5.7	6.15±0.77

LC_{50} of yellow fruit toward T47D cell after 24 hours incubation is 0.25 µL/mL. According to Ueda et al*.* (2002) the extract of plantation with LC_{50} < 100 g/mL is potential to be developed as anti cancer. In this experiment, DMSO was used as solvent despite of water since the extracted compound was difficult to dissolve in water. Nogaki et al. (1998) reported that DMSO does not disturbing the growth of HL-60 cell and HSC-40, so that it can be used as solvent. We assumed that 0.7% DMSO used in this experiment will not significantly influence percentage of life and morphology of T47D cell.

Based on Figure 1. Significant effect of the yellow fruit extract on T47D death cell was observed.

Correlation between extract concentration and the level of of expression of *p53* mutant is shown with r value of 0.967. This figure shows that the extract concentration under LC_{50} inhibited the level of expression of *p53* significantly. This possibly caused by the excistance of the bioactive compound (tocopherol, β-carotene and carotene) in the extract which inhibit proliferation of T47D cells. Research by Carlisle et al*.* (2000) found that tocopherol could induce apoptosis and promote the expression of *p53* in the lung cancer (HLF cell). Moreover, the combination of tocopherol and vitamine A shown that it could inhibit the growth of metastasis in breast cancer cell in transgenic experiment (Albright et al*.,* 2004). The activition of the *p53*-wildtype might happen due to the reactivation of *p53* mutant. Beside this, there is also possibility that the reactivation of biological function of *p53*-wtp which was done by some compound in the yellow fruit.

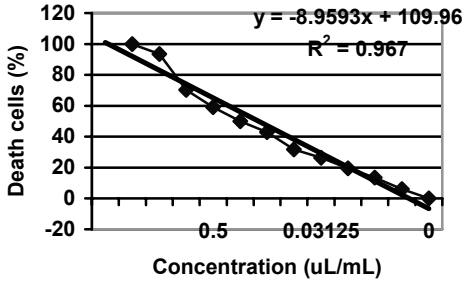

Figure 1. The effect of the crude extract of *P. conoideus* var. yellow fruit on death of T47D cell.

In general, the treatment with yellow fruit extract could significantly inhibit the growth of T47D cell as indicated by doubling time test (Table 2). Addition extract of yellow fruit with dosis of 0.125 uL/mL made a reaction the dubling time from 20.362 hours become 37.989 hours (1.86 time), while at the concentration of 0.0625 µL/mL and 0.03125 µL/mL its reaction time longer and the value of doubling time become 27.376 hours (1.34 times) and 23.220 hours (1.14 times).

Table 2. The regression similarity between number of cells in certain incubation time and the doubling time.

Material	Concentr a-tion (µL/mL)	The line equation of the incubation time versus the amount of cells	Slope value	R^2	Doubling time value
Crude extract of *P. conoideus* var. yellow fruit	Control	Y=0.1988x+0.562	0.1988	0.923	20.322
	0.03125	Y=0.159X + 0.91	0.159	0.929	23.220
	0.0625	Y=0.125X +1.18	0.125	0.944	27.376
	0.125	Y=0.088X +1.259	0.088	0.899	37.989

The discovery of the compound potential as cytostatic agent for *p53* is very important in the effort of healing cancer. This is due to the cell cancer in human being is usually caused by abnormal function of the *p53*, so that the growth of cancer could be inhibitted by applying such compound.

Based on the promoting effect of cytostatic due to the addition of yellow fruit extract, there is a possibility of activating *p53*-wildtype so that it will be the process of apoptosis. Nuclear fragmentation as marker of apoptosis in a microscopic scale show increased in the treatment of the extract in the concentration close to the LC_{50}.

Apoptotic test to know the effect of the yellow fruit extract in inducing apoptosis in T47D cells wad carried out by TUNEL enzymatic assay staining, and observed under the fluorescens microscope. The positive apoptosis cells will be in bright green color, whereas the viable cells will be in orange color (Rode et al., 2004). Both viable cells and apoptotic cells are presented in Figure 2. TUNEL is a quick method to identify and know the cells quantity which getting apoptosis in cell culture treatment (Wyllie, 2000; Wieder, 2005; Darzynkiewicz et al*.,* 2008).

Decreasing amount of viable cells after treated by yellow fruit extract indicated the blocking of cell proliferation that occurs related to the decreasing of protein synthesis as needed in the process of proliferation. The TUNEL enzymatic assay staining was employed to evaluate morphological change of T47D. The cells that undergo apoptosis appear as green colour, while the cells that experiencing the first apoptosis, their plasma membrane is still intact and will be in orange colour, but begin to form the chromatin condensation that resulting in green spots. It thus can be diferentiated between viable and necrotic cells. The percentage of apoptosis of T47D cells after 24 hours incubation with yellow fruit extract is presented in Table 3.

Table 3. The average percentage of apoptosis of T47D cells after 24 hours incubation with crude extract of *P. conoideus* var. yellow fruit.

Concentration (µL/mL)	Apoptosis (%±SD)
0.125	34.38±2.26
0.0625	30.03±3.87
0.03125	21.07±1.14

The death cells include (i) apoptosis,the progammed cells death that physiologically to balance and it is marked by DNA fragmentation, chromatin condensation, decreasing the cells size, cytoplasmic prominent and forming apoptosis body (ii) necrosis, death cells pathologically bringing of inflamation (King, 2000).

The *p53* protein code by *p53* gene is located in short arm of 17st human chromosom. Two types of *p53* protein is recognized i.e *p53* protein wild type and mutant type. The amount of *p53* protein wild type in nucleus is in small amount, labile and has short half life time so it could not be detected by immunohistochemical staining technique. This protein

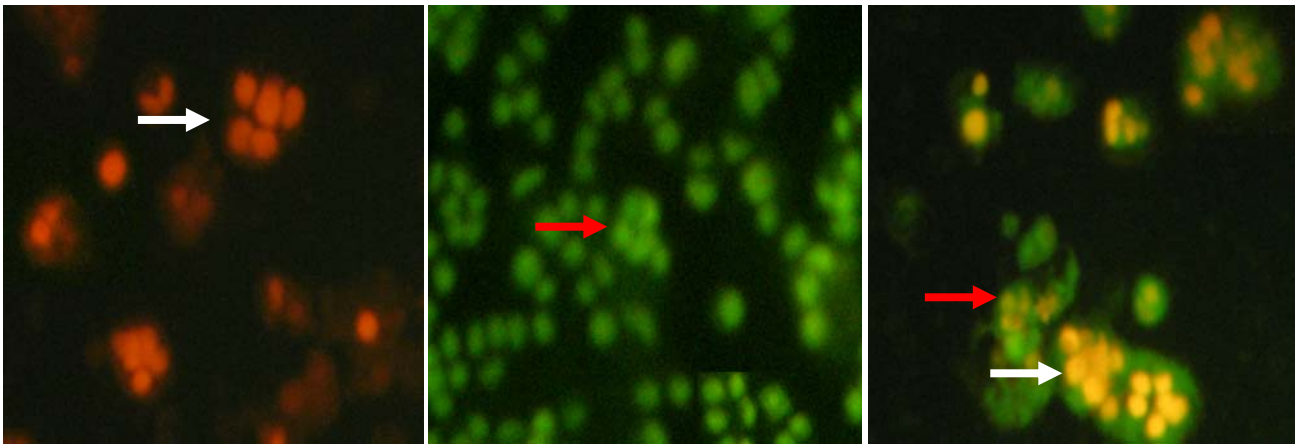

Figure 2. T47D cells after the treatment of crude extract of *P. conoideus* var. yellow fruit stained by TUNEL *enzymatic assay*. A. Negative control, B. Positive control of T47D cells undergo apoptotic (red arrow), C. Viable T47D cell (white arrow).

contribute to block cells proliferation, transcription, DNA repair and apoptosis, whereas *p53* protein mutant type contribute in blocking the *p53* protein wild type until cells proliferation loss its resistance (Brock, 1993).

Controling cell cycle is done in order to perform a normal cycle. Cdk (*cyclin dependent kinase*) like Cdk 4 Cdk 6, Cdk 2 along with *cyclin* (*cyclin* D, *cyclin* E, *cyclin* A and *cyclin* B) are main substances involved in cells cycle, which bringing on the movement from G1 to S or from G2 to M (Guardavaccaro et al*.,* 2000). MPF (*Maturation Promoting Factor*) along with Cdk and cycling become progressively trigger the cell cycle. The *p53* protein function as blocking of cell cycle if DNA damage taken place, and if a serious damage occurs could resulted in an apoptosis (Brown and Wouters, 1999).

CONCLUSION

The crude extract of *Pandanus conoideus* Lamb. var. yellow fruit is potential as anti cancer. The results of the research evidence that the LC_{50} of yellow fruit extract are 0.25 µL/mL. The percentage of apoptotic in the dosage of 0.125 µL/mL, 0.0625 µL/mL and 0.03125 µL/mL are 34.38±2.26, 30.03±3.87 and 21.07±1.14 respectively.

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