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Yeast *Saccharomyces cerevisiae* as Model to Identify Mengkudu (*Morinda citrifolia*) as an Anticancer Medicinal Plants Candidates with Antiproliferative Properties

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Although yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model organism, however, to use *S.cerevisiae* cell as a model organism screening drugs is rare. In this research, we used *S. cerevisiae* to screen antiproliferative properties of mengkudu or noni fruit (*Morinda citrifolia*). The results showed that 3% w/v extract of mengkudu, but not pare and sukun extracts was capable to inhibit growth of *S. cerevisiae* effectively. This inhibited phenotypes was confirmed by measuring growth rate of cells in cultures treated with various concentrastions of extracts. Morphologycal analysis using differential interference contrast (DIC) microscopy showed that *S. cerevisiae* wild type strain FY833 cells was accumulated in the unbudded cells, and further analysis using Fluorescence Activated Cell Sorting (FACS) analysis exhibited that proliferation of cells run unproperly because of cells are arrested in G1 phase, indicating that G1 to S phase transition progression of *S. cerevisiae* cell cycle was inhibited in the presence of mengkudu extract.

Keywords: *Saccharomyces cerevisiae, mengkudu, anticancer antiproliferative*

1. Introduction

At least 5,596 genes encoding protein of yeast *Saccharomyces cerevisiae* has been completely sequenced (Otero *et al*, 2010). Therefore, the budding yeast *S.cerevisiae* is being widely used as a model for investigating fundamental celluler mechanisms relevant to all living organisms. The biochemical and physiological functions of higher eukaryotic genes could be studied from *S.cerevisiae* genes function based on their functional analogies (Foury, 1997). Some advantages using *S.cerevisiae* are simple to culture in large quantities, and the cell cycle can be easily manipulated both genetically and physiologically.

The budding yeast *S.cerevisiae* is great help in understanding the celluler function of disease. Many crucial proteins in human were iniatially discovered by studying their homologs in yeast. Many of disease mechanism are affected by genetic and epigenetic alterations in cancer such as cell cycle progression, DNA replication and segregation, maintenance of genomic integrity and stress responses. Therefore, yeast emerges as an attractive model for anticancer drug research (Menacho-Marquez and Murguia, 2007).
The cell cycle is a series of events involving the growth, replication, and division of a eukaryotic cell. Replication of DNA occurs in a specific part of the interphase called S phase. S phase is preceded by a gap 1 phase (G1) during which the cell is preparing for DNA synthesis and is followed by a gap 2 (G2) during the cell prepares for mitosis (M). In normal cell, transition progression of cell cycle in each G1, S, G2, and M phases is regulated tightly (Vermeulen *et al*, 2003; Alberghina *et al*, 2010). In cancer, there are fundamental alterations in the genetic control of cell division, resulting in an uncontrolled cell proliferation. There is defect in a regulation of the cell cycle. Key regulatory proteins of cell cycle progression are cyclins and cyclin dependent kinases (CDK). *S.cerevisiae* is powerful tool for investigating the molecular regulation of cell cycle control.

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In this research, our goals are to check whether traditional medicinal plant *mengkudu* or *noni fruit* (*Morinda citrifolia*) (Bangun, AP, 2002) affect the growth progression of *S.cerevisiae* which indicating as antiproliferative and anticancer drug, and to use an excellent model organism, a normal cell *S.cerevisiae* wild type strain FY833 as preclinical assay for antiproliferative. To my knowledge, publications using of normal cell budding yeast *S.cerevisiae* as anticancer drug discovery for identification and characterization of traditional medicinal plant is rare.

The results showed that 3% w/v extract of *mengkudu* was capable to inhibit growth of *S.cerevisiae* effectively. Furthermore, based upon morphologycal analysis showed that higher concentration of *mengkudu* extract was added, higher percentage of unbudded cells. Consistent with this result, Fluorescence Activated Cell Sorting (FACS) analysis also showed that in the presence of 3% (w/v) *mengkudu* extract *S.cerevisiae* wild type strain FY833 cells was accumulated in the unbudded cells, indicating that the cell cycle growth progression of *S.cerevisiae* was inhibited in G1 to S phase. Cells with enlarge of nuclear were also observed in the presence of that *mengkudu* extract. Taken together, these results indicated that *S.cerevisiae* is a promising tool for studying identification of antiproliferative character of traditional medicinal plant candidate.

2. Experimental

Extraction using methanol

Mengkudu (*M.citrifolia*) dried sample (25 g) was extracted by soxletation method using methanol solvent. The extract is concentrated using rotary evaporator under vacuum at room temperature to obtain a final residue for further assay.

Spot assay experiment

S.cerevisiae strains BY5209 (= FY833) (Winston *et al*, 1995) with genotype *MAT***a** *ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63* cells grown in YPAD medium (consists of 1% bacto yeast extract, 2% bacto pepton, 2% dextrose, 40 mg/100 ml media adenine sulphate) to mid logarithmic phase, suspensions containing equal cell-numbers were prepared on the basis of OD_{660} and ten-fold serial dilutions were spotted onto YPAD plates supplemented with 3% w/v of *mengkudu* methanol extract that were incubated for one to two days.

Growth curve

Fresh media (YPAD, YPAD with 3% w/v *mengkudu* methanol extract) was added overnight culture to a cell density of OD_{660} 0.1. Cultures of *S.cerevisiae* were grown at 30°C and 145 rpm. Cell growth were measured as OD_{660} every 2 hours until reach or stationary phase.

Morphology observation

Cells grown in YPAD and YPAD with 3% w/v *mengkudu* methanol extract) to an OD_{660} = 1.0 were concentrated twenty-fold in sterilezed water. Then immediately analyzed with a fluorescence microscope (BX61-34-FL-I-D, Olympus) using a BF and DAPI filter (Olympus), CCD camera (CCD-Exi, Molecular Devices) and MetaMorph version 6.1 software (Molecular Devices).

Synchronization of cell cycle

Cells grown in YPAD to mid logarithmic phase were arrested in G1 phase by exposure to 2 μg/ml α-factor (Sigma) for three to four hours, after which cells were washed five times with YPAD and transferred into fresh medium without and with methanol extract

3% (w/v) *mengkudu* extract. At least 500 cells were counted to determine the percentage of budded cells.

FACS (Fluorescent-Activated Cell Sorting) analysis (Haase and Lew, 1997).

An 0.25 ml aliquot of yeast cells (OD_{660} = 1.0) grown in YPAD broth without and with methanol extract 3% (w/v) *mengkudu* extract was harvested by centrifugation, and, after being resuspended by gentle vortexing, fixed with cold ethanol (-20°C) for at least 12 hours. Washed twice with 0.2 M Tris-HCl pH 7.5, and sonicated with minimum output (set the level “1”) for 5 second (2 second x 2 times) with interval. Cells were harvested by centrifugation for 5 min at 15,000 rpm and resuspend in 100µl of Rnase solution (1 mg/ml RnaseA in 0.2 M Tris-HCl pH 7.5) at 30°C for 3-4h. Cells were pelleted and resuspended in the dark with 300 µl of 0.2 M Tris-HCl (pH 7.5) with 100 µg/ml propidium iodide on ice, added 400 µl of 0.2 M Tris-HCl pH 7.5, chill on ice until measuring DNA contents. Filter suspension cell used 5 ml polystyrene round bottom tube within cell stainer cap, then subjected to FACS analysis (Epix XL, Beckman Coulter)

3. Results and Discussion

***Mengkudu* extract indicates antiproliferative**

Traditional medicinal plant candidate sample (*mengkudu*, *pare*, and *sukun*) were successfully extracted by soxletation using methanol solvent. Antiproliferative character initially was tested sensitivity of cells for samples extract by spot analysis in both YPAD agar media and YPAD broth media with various concentrations 0% - 5%(w/v) *mengkudu* extracts. Eventhough in the presence 5% (w/v) concentration of *pare* extract and *sukun* extract, *S.cerevisiae* cells could grow as well as in the absence of those extracts (Figure is not

shown). Opposite with above result, in the presence of mengkudu extract, in YPAD agar medium, cells are clearly sensitive phenotype in the presence of 4%(w/v) *mengkudu* extract. While using YPD broth medium, growth curve of *S.cerevisiae* cells showed cells capability to grow, the result indicated that the growth is weaker from 0% to 3% (w/v) *mengkudu* extract-present, and from in the presence of 3% to 5% (w/v) cells capability to grow is completly no growth (Figure 1 and Figure 2).

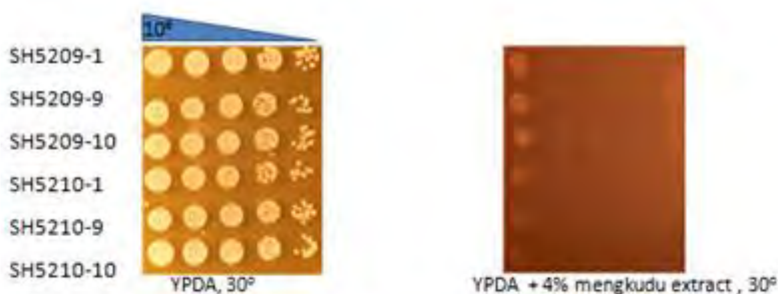

Figure 1. Spot analysis of S.cerevisiae for *mengkudu*, *pare*, and *sukun* extract. In the presence of 4%(w/v) of mengkudu the cells are sensitive.

The growth of cells especially in presence of mengkudu extract, exponential phase of growth was not clear in the higher concentration of extract, indicating that inhibited growth of cells was occured, indicating that mengkudu extract had antiproliferative for *S.cerevisiae* cells. Wang and Su (2001) described that *mengkudu* has broad therapeutic effects including anticancer activity, in both clinical practice and laboratory animal models, although the mechanism for these effects remains unclear. But, hypothesis mentioned that mengkudu possesses a cancer

preventive effect at the initial stage of carcinogenesis, this hyphotesis was studying furthermore.

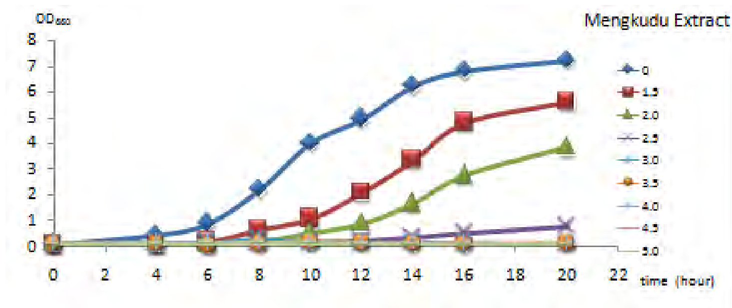

Figure 2. Growth curve *S.cerevisiae* in various concentrations (0-5% w/v) of *mengkudu* extract In Figure a shows in the presence of *mengkudu* extract 0-3%(w/v) growth of cells is slower, while 3-5%(w/v) growth almost stopped.

Unbudded cells are more than budded cells

Since in previous result showed that mengkudu extract but not pare or sukun extracts could inhibit the growth of *S.cerevisiae* cells, we observed form of cells in cultures when cells have been incubated for 4 hours with iniatially optical density cells (OD_{660}) 0.1. At least 500 cells were observed their form, and catagorized into two groups, budded cell and unbudded cell. Result showed that higher concentration of mengkudu extract, more unbudded cells for, indicating higher mengkudu extract could prevent cells forming a budding (Figure 3). Unbudded cell form indicated that cells are in the G1 phase, while budded cell with short spindle form indicated that cells are in the S+G2 phase, and budded cell with long spindle indicated that cells are in the M phase (Nakashima *et al*, 2008).

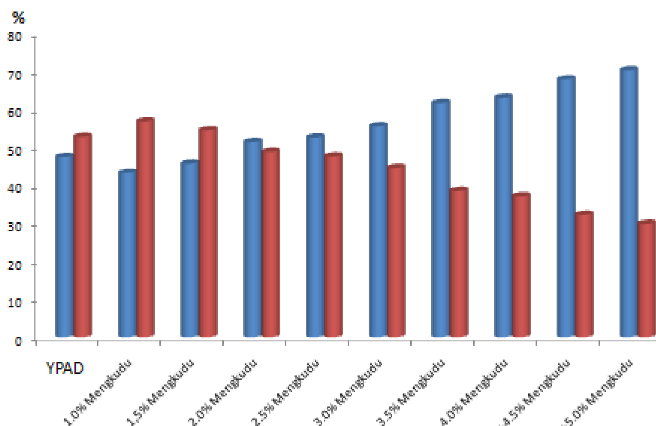

Figure 3. Comparison of budded cells percentage and unbudded cells percentage. In the presence of *mengkudu* extract induced inhibited growth of budding. At least 500 cells grown to mid log phase were checked. Blue bar indicates unbudded cells form, and red bar indicates budded cells form.

FACS analysis revealed that G1 to S transision was arrested

To confirm the inhibited growth of *S.cerevisiae* cells was treated with mengkudu extract, we conducted FACS (fluorescence activated cell sorting) analysis.

FACS analysis is one of the most useful tools to study cell cycle progression in budding yeast by monitoring their DNA content (Qaddouri *et al*, 2009).

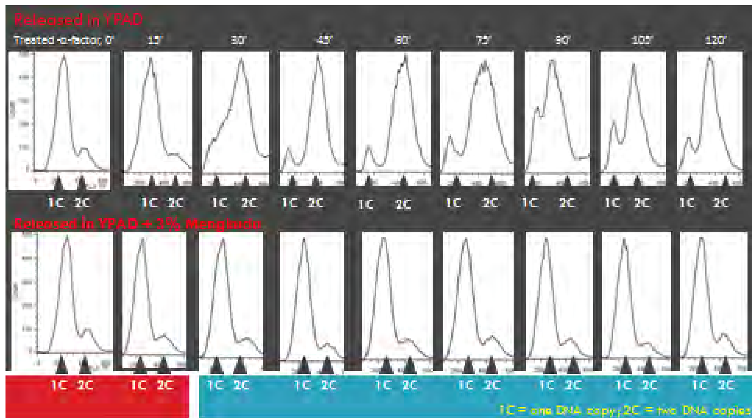

Figure 4. FACS analysis. 3%(w/v) *mengkudu* extract unbudded cells (1C) inhibited G1 to S progression. 1C, one DNA copy; 2C, two DNA copies.

First, *S.cerevisiae* cells were arrested using the mating pheromone alpha (α) factor to get synchronized cells in G1 phase (Futcher, 1999). Effect of *mengkudu* extracts to the *S.cerevsiae* cell cycle were observed after 90 minute release into broth media containing each of those extracts. In the presence of mengkudu extract as in Figure 4b S.cerevisiae cells were arrested in G1 phase, indicated that progression transition of G1 to S is not run properly. This make proliferation or increasion of cell number of *S.cerevisiae* cells are stopped.

Enlarge nuclear of cells

Observation of *S.cerevisiae* cells using fluorecence with DAPI (4',6-diamino-2-phenylindole) staining and differential interference contrast (DIC) microscopy was carried out to monitor not only cell cycle progression but also to monitor morphological cell whether extracts induced cell alteration. The result showed that cells treated with mengkudu having an enlarge nuclear compared to cells untreated (Figure 5). Some chemicals such as $CaCl_2$ causing

fragmented vacuolar cell (Hermansyah *et al*, 2009; 2010) or cells treated with Lyc, a plant-derived natural alkaloid product showing cells typically had a fragmented nucleus (Qaddouri *et al*, 2009).

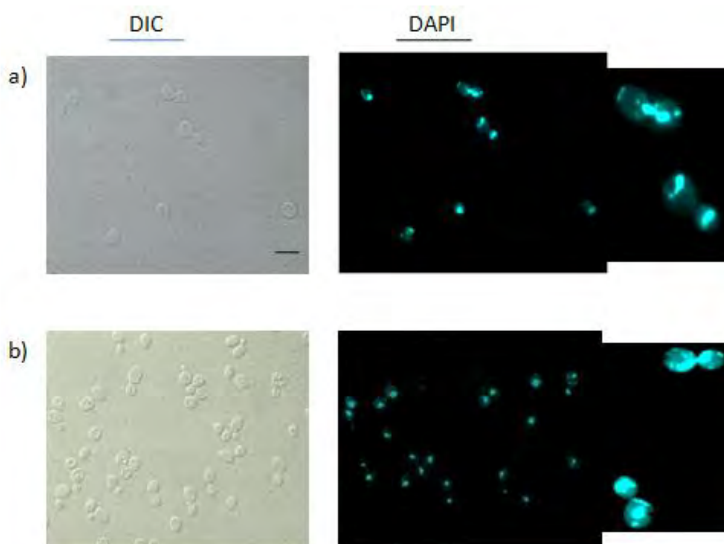

Figure 5. Comparison of morpholical cells, cells were grown in YPAD without and with *mengkudu* extract. Figure shows enlarged nuclear in cells treated with mengkudu extract. Bar 1µm.

4. Conclussions

This research exhibits usage of budding yeast *S.cerevisae* as a useful model oragnism to discover an anticancer drug and identification the antiproliferative properties of tradional medicinal plants candidate. Growth of *S.cerevisiae* cells was clearly inhibited in the presence of mengkudu extract. FACS analysis revealed that inhibited growth of *S.cerevisiae* cells which treated with mengkudu because of arrested G1 phase.

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