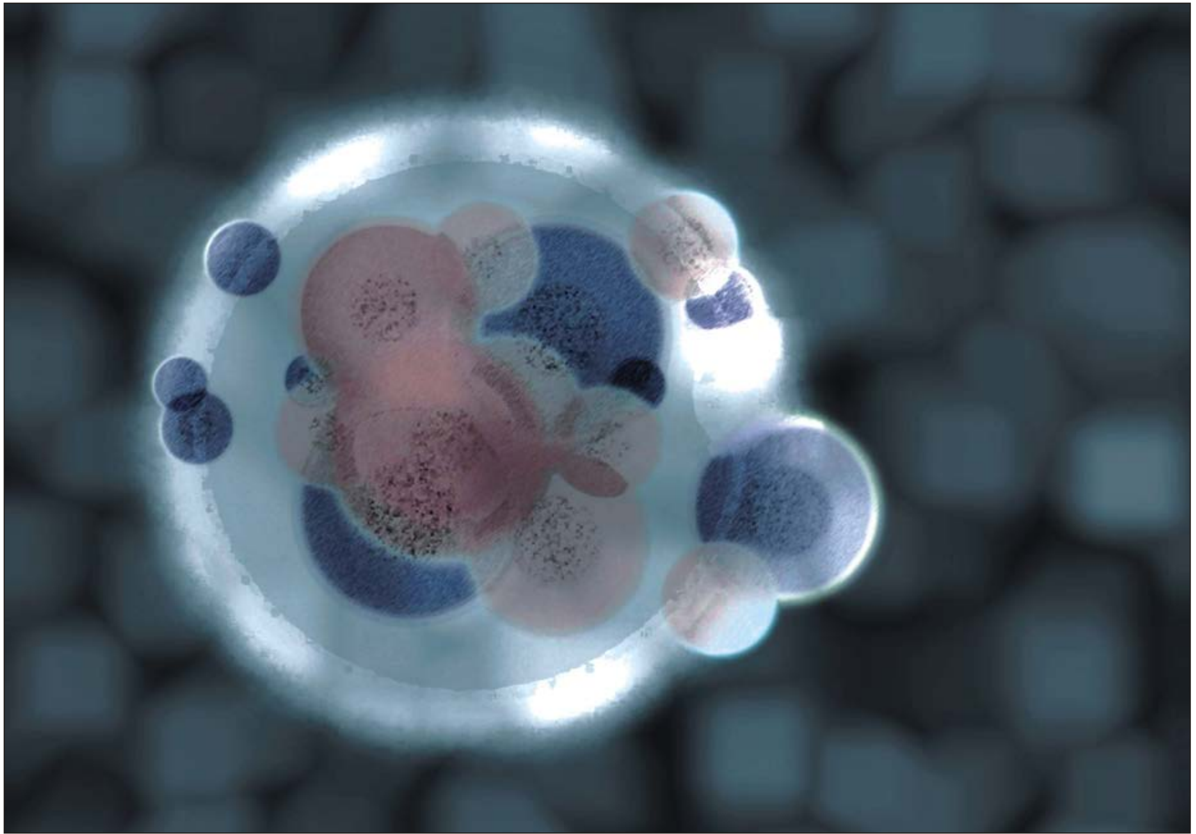

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Antioxidant Activity of Flavonoid from *Guazuma ulmifolia* Lamk. Leaves and Apoptosis Induction in Yeast Cells

[1]Syaefudin, [2,3]W.T. Wahyuni, [1]I.M. Artika and [1,3]Sulistiyani
[1]Department of Biochemistry, Bogor Agricultural University, Bogor, 16680, Indonesia
[2]Department of Chemistry, Bogor Agricultural University, Bogor, 16680, Indonesia
[3]Biopharmaca Research Center, Bogor Agricultural University, Bogor, 16128, Indonesia

Abstract: Antioxidant activity was considered to be correlated with atherosclerosis prevention through apoptosis modulation. *Guazuma ulmifolia* Lamk. has been reported as potential antioxidant, however the potency of its flavonoid as apoptosis modulator has not been investigated. The research objective was to investigate the antioxidant activity and the effect of Guazuma's flavonoid on the growth of yeast cells. Antioxidant activity of *Guazuma ulmifolia* Lamk. leaf was determined by thiobarbituric acid (TBA) assay. The cellular effect was investigated using *Saccharomyces cerevisiae* system. Results showed that antioxidant activity of the flavonoid at 100 ppm was higher than α-tocopherol 200 ppm (88.52%). Flavonoid was found to induce petite colony formation from 75% of the total colonies and induced membrane blebbing of yeast cells indicating apoptotic events. There was lesser colony growth (>30% reduction) in flavonoid-treated cells than in those treated with 4% glucose as control, suggesting that the flavonoid from *Guazuma ulmifolia* Lamk. leaves could induce apoptotic events on yeast cells. Guazuma's flavonoid has inhibitory effect on cell proliferation, indicating the effect of flavonoid on other mechanisms in cellular activities.

Key words: Antioxidants, flavonoid, apoptosis, membrane blebbing, *Guazuma ulmifolia*

INTRODUCTION

Guazuma ulmifolia Lamk. (Guazuma) leaves extract has been reported to reduce blood lipid concentration and inhibited the occurrence of atherosclerosis in hyperlipidemic animals (Sukandar *et al*., 2012). The significance of the modulation of apoptosis in the event of atherosclerosis progression has only been addressed recently (Tabas, 2005). It has been proposed that apoptosis is modulated by oxidative stress and that antioxidants play a part in its prevention (Chandra *et al*., 2000). Thus, antioxidants may play a role by modulating (induction or inhibition) the occurrence of macrophage cell apoptosis (Moore and Tabas, 2011; Tabas and Ron, 2011). Guazuma leaf extract has been reported for its antioxidant activity. Antioxidant activity of Guazuma leaf extracts was reported to be higher than α-tocopherol (200 ppm) (Syaefudin, 2008), nevertheless the antioxidant activity of Guazuma's flavonoid and its role in inducing apoptosis has not been properly investigated.

This study aimed to determine the antioxidant activity of Guazuma's flavonoid fraction and its role in inducing apoptosis in yeast cells as a model. Yeast cells that undergo apoptosis could be detected by observing the formation of 'petite cells' (cell shrinkage) and the blistering of the membrane (membrane blebbing) (Kerr *et al*., 1972; Hacker, 2000; Granot *et al*., 2003; Ziegler and Groscurth, 2004; Henry *et al*., 2013).

MATERIALS AND METHODS

Flavonoid fractionation of *Guazuma ulmifolia* Lamk. leaf: Guazuma leaves were extracted by using Mabry method (Mabry *et al*., 1970). A total of 100 g leaf powder (Biopharmaca Research Center, Bogor, Indonesia) was macerated in methanol/H_2O 1:1 for 24 h to produce 50% methanol filtrate. Similarly, another 100 g of leaf powder was macerated with methanol/H_2O 9:1 to produce 90% methanol filtrate. Each was repeated 3 times. Both filtrates (50 and 90%) were mixed and then concentrated to obtain a less aqueous methanol/H_2O extract. Subsequently, the extract was partitioned using n-hexane that gave n-hexane fractions and residue. Then, the residue was fractionated with chloroform to obtain flavonoid extract. This flavonoid extract will be diluted in 50% ethanol. All chemicals for solutions were purchased from Merck, Darmstadt, Germany.

Corresponding Author: Syaefudin, Department of Biochemistry, Bogor Agricultural University, Bogor, 16680, Indonesia
Tel/Fax: +6251-8423267

Malondialdehyde (MDA) concentration analysis by TBA method: Standard curve was made by diluting 1, 1, 3, 3-tetramethoxypropane (TMP) 6 M (Merck, Darmstadt, Germany) to various concentrations series. Sample mixtures which consisted of 2 mL phosphate buffer 0.1 M pH 7 (Merck, Darmstadt, Germany), 2 mL linoleic acid 50 mM (Sigma-Aldrich GmbH, Steinheim, Germany) in 99.8% ethanol and 1 mL of extract (dissolved in 50% ethanol) were prepared. Blanks were prepared similar to sample mixture, except that the extract was substituted with deionized water. Control for antioxidant activity was prepared by mixing 2 mL of phosphate buffer 0.1 M pH 7 with 2 mL of linoleic acid 50 mM in ethanol 99.8% which contained α-tocopherol (Sigma-Aldrich GmbH, Steinheim, Germany) in final concentration of 200 ppm and 1 mL of deionized water. All mixtures except the standards were incubated in the dark using water-bath at 40°C for 8 days prior to MDA determination. The samples were incubated in a water bath at 40°C for 8 days. One millilitre of each sample was taken and was added with 2 mL trichloroacetic acid (TCA) 20% (Merck, Darmstadt, Germany) and 2 mL thiobarbituric acid (TBA) 1% (Merck, Darmstadt, Germany) in 50% acetic acid (Merck, Darmstadt, Germany) and mixed thoroughly. Sample mixtures and standards were placed in a hot water bath at 100°C for 10 min. After cooling and centrifugation (Hettich Zentrifugen D-7200 Tuttlingen 1200, Germany) at 3000 rpm for 15 min, the absorbance of the samples was measured using spectrophotometer (Thermo Electron Corporation Genesys 10 UV, USA) at wavelength of 532 nm (Kikuzaki and Nakatani, 1993).

Role of flavonoid extract in apoptosis induction of yeast cells

Cells regeneration and maintenance: Yeast cells were obtained from IPB Culture Collection (IPBCC), Biology Department, Bogor Agricultural University. For cell regeneration, two colonies of cells were grown on solid medium of Yeast Extract Peptone Dextrose (YEPD) which composed of 1% yeast extract, 2% peptone, 2% glucose and 1.8% bacto agar. All culture media were purchased from Difco Laboratories (Becton, Dickinson and Company, New Jersey, USA). Cells were incubated at 28°C for 2 days. After that, cells were ready to be used or stored in a refrigerator (8°C) (Campbell and Duffus, 1988).

Yeast cells apoptosis induction: An ose of yeast cells which had been rejuvenated was transferred into 10 mL YEPD liquid medium and placed in a waterbath shaker (MMS 3010 Tokyo Rikakikai Co. Ltd., Tokyo, Japan) (121 rpm) for 24 h at room temperature (28°C). From this 200 μL of cell cultures were transferred into 20 mL YEPD liquid medium. Cell cultures were incubated further at 28°C for 48 h or until the reading of Optical Density (OD) at wavelength of 600 nm measured between 1.355-1.458. Subsequently, cell cultures were centrifuged Hettich Zentrifugen D-7200 Tuttlingen 1200, Germany) at 3000 rpm for 15 min at low temperature (4°C) and washed twice by 15 mL sterile distilled water. Groups of cell pellets were then incubated with (1) Flavonoids in ethanol, (2) Glucose 4%, (3) Ethanol (final concentration 1%) and (4) YEPD medium (normal cell control). Except normal control cells which were incubated at 28°C, other cell cultures were incubated at 37°C for a period of 24 h (Granot *et al*., 2003).

Viable cells and 'petite cell' frequency test: After 24 h incubation, treated cells were diluted to 10^{-6} and 200 μL aliquots were distributed on normal YEPD medium or petite YEPD medium. The composition of petite medium consisted of 1% yeast extract, 2% peptone, 0.1% glucose and 1.8% bacto agar. After incubation at 28°C for 24 h, cells which were growing on each petri dish were classified as petite cells for smaller cells and larger cells as normal cells. Petite cells frequencies were calculated using the equation:

$$\text{Petite cells frequencies} = \frac{\text{Petite cells}}{(\text{Petite cells+normal cells})} \times 100\%$$

The total colonies of yeast grown were counted as viable cells.

Cells morphological examination by scanning electron microscopy (SEM): Samples were fixed by 2% glutaraldehyde, centrifuged and the supernatant was discarded. Tannic acid 2% was added to the samples and soaked for 12 h. After centrifugation, the fixative solvent was discarded and pellets were added with cacodylic buffer. After soaking for 2×10 min, the samples were centrifuged and the buffers were discarded. The samples were added with 1% osmium tetroxide and soaked for 1 h, followed by centrifugation and the supernatants were discarded. The remaining samples were soaked twice in 50% alcohol for 10 min followed by alcohol 70, 80 and 95% washing, every 10 min. Finally, absolute alcohol was added twice for 10 min. Samples were centrifuged and the solvents were discarded. Samples were soaked twice in t-butanol for 10 min, followed by centrifugation. After discarding the solvent, samples were resuspended in butanol and applied on to the frozen cover slip, dried and scanned with SEM (Jeol JSM 5310-LV, Tokyo, Japan).

Statistical analyses: Data were analyzed using ANOVA to determine significant differences ($p \leq 0.05$). Multiple

comparisons were done by the Tukey HSD and Duncan tests. All statistical analysis were done with the software SAS (SAS Institute, Inc. Cary, NC, USA) version 9.1.3 and SPSS (SPSS Inc. Chicago, IL, USA) version 13.

RESULTS AND DISCUSSION

Antioxidant activities: Linoleic acid incubation with excess oxygen resulted in increased production of malonaldehyde (MDA) (20.62 μm). The addition of α-tocopherol (200 ppm) into the system inhibited the formation of MDA up to 71.57%. This effect was consistent with other studies, which reported that α-tocopherol could prevent oxidation on lipid (Halliwell and Chirico, 1993; Pekiner, 2003). Lusiana (2010) reported that α-tocopherol (200 ppm) could inhibit the formation of MDA as much as 66.47%. In this experiment, flavonoid fraction of *Guazuma* leaves showed antioxidant activity as much as 88.52% (Fig. 1). Duncan statistical test showed significantly higher antioxidant activity of 100 ppm extract, compared to that of α-tocopherol (200 ppm). At the concentration of 50 ppm, however, the antioxidant activity of the extracts was not significantly different as compared to α-tocopherol (α = 0.05). Other research on stem bark of Guazuma found that its flavonoids have strong free radical scavengers (Feltrin *et al*., 2012).

Effect on growth of yeast cells

Induction of petite colony: The yeast cells which were induced by 4% glucose were smaller in size (petite) compared to normal cells (Fig. 2). This finding was consistent with study by Lusiana (2010) which resulted that yeast cells incubated in media with high glucose concentration showed increased petite cell frequency. The petite colonies of yeast cells caused by high glucose concentration is an indication of apoptosis events (Granot *et al*., 2003). The small sized cells were due to shrinkage volume of cell which was one of sign of apoptosis (Klassen *et al*., 1993; Chang *et al*., 2000; Friis *et al*., 2005). A similar cell shrinkage also occurred in yeast treated with flavonoid. The size of petite cells resulted from extract induction were not much different from that induced by glucose at 4%.

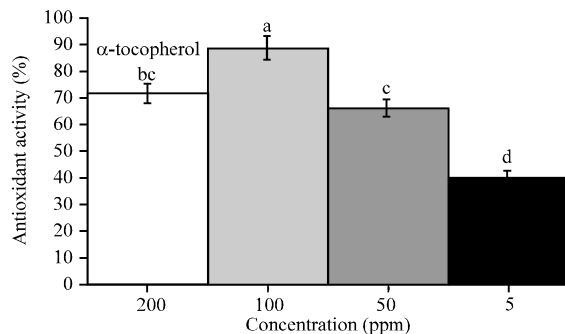

Fig. 1: Antioxidant activity of *Guazuma ulmifolia* at various concentrations. The same letter on the bars indicated statistical non-significance (p>0.05)

Petite colony frequency: The incubation of yeast cells with glucose induced petite colonies with an increased frequency up to 76.50%. Normal cells had much lower petite frequency (8.54%) than the cells which were induced by glucose 4% (Fig. 3). Similar observation was found on yeast cells treated with flavonoid of *Guazuma*; cells incubated with 100 ppm flavonoid fraction had a petite colony frequency up to 74.52%. Duncan statistical test confirmed that both plant fractions and glucose had the same ability to induce petite colonies. Higher concentration of flavonoid (100 ppm) induced greater frequency of petite colonies than the lower 50 ppm. These results were consistent with the study which showed that

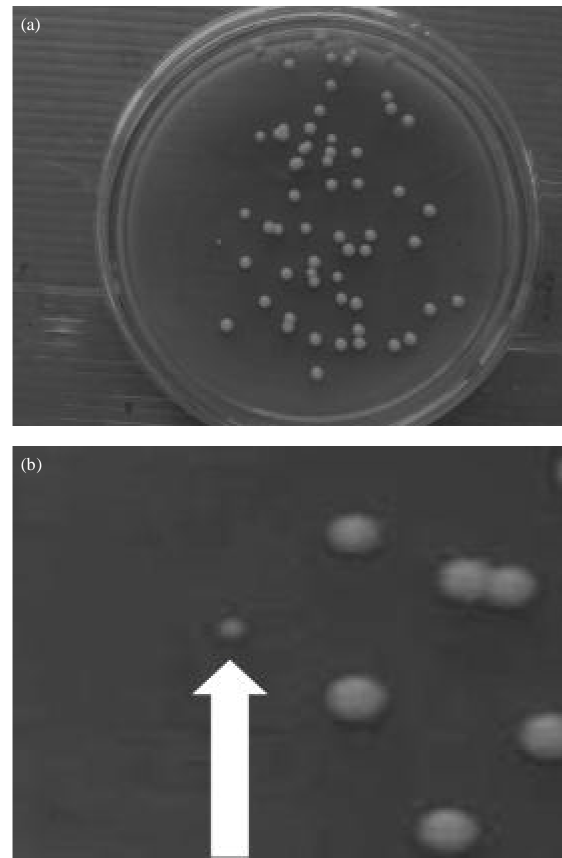

Fig. 2(a-b): (a) Cultures of *S. cerevisiae* colonies and (b) Petite colonies (marked with white arrow)

the higher concentration of the *Guazuma*'s crude extract caused a greater petite frequency (Lusiana, 2010). The ability to induce greater frequencies of petite colonies seems to be proportional to its antioxidant activity.

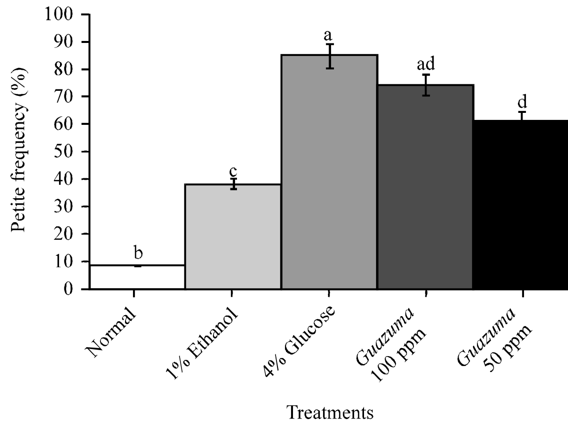

Fig. 3: No. of petite yeast cells on various treatments. The same letter on the bars indicated statistical non-significance (p>0.05)

Membrane blebbing: Upon examination with Scanning Electron Microscope (SEM), normal yeast cells were shown as large sized cells with smooth surface and rounded and or oval shaped and there were some cells budding. In contrast, glucose induction caused yeast cells to have rough surface and shrunken cell shape, small sized cells, grooved and blebbing (Fig. 4). Treatment of cells with flavonoid also produced smaller sized cells with similar shape and the morphology of yeast cells treated with 4% glucose were characterized by the presence of membrane blebbing. Membrane blebbing is one characteristic of yeast cells undergoing apoptosis. Besides the smaller size than the normal cells, the cells which were undergoing apoptosis also had condensed cytoplasm and cell organelles (Elmore, 2007). These observations were similar with the induction of 2% glucose which caused apoptosis in yeast cells, characterized by blistering of cell membranes (Granot *et al.*, 2003). The blistering of yeast cell membranes was due to increased caspase enzyme activity during apoptosis (Mills *et al.*, 1998; Coleman *et al.*, 2001).

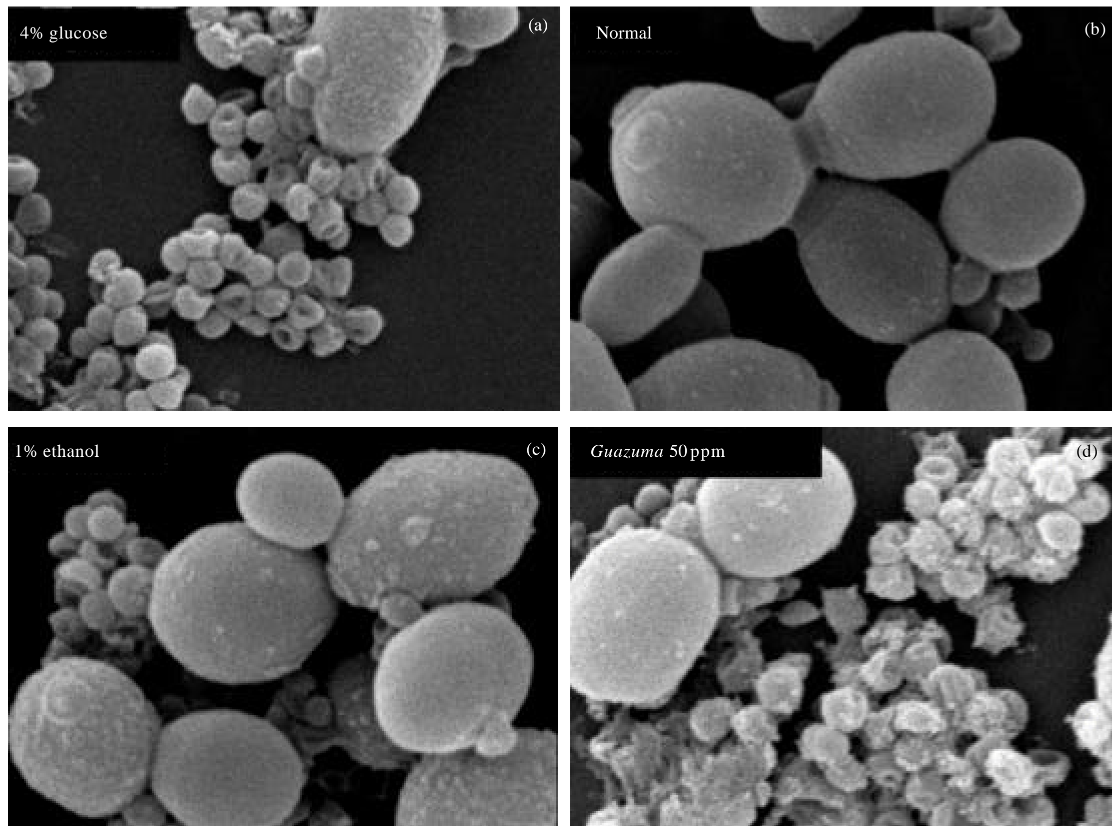

Fig. 4(a-d): Morphology of yeast cell by SEM (20 kV, 10.000×) (a) 4% glucose (b) Normal (c) 1% ethanol and (d) *Guazuma* 50 ppm

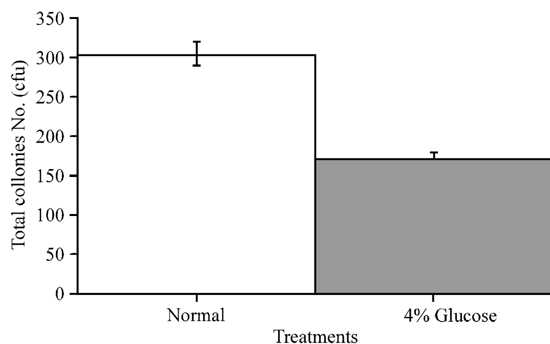

Fig. 5: No. of viable colonies of yeast cells. Control cells and 4% glucose-induced cell

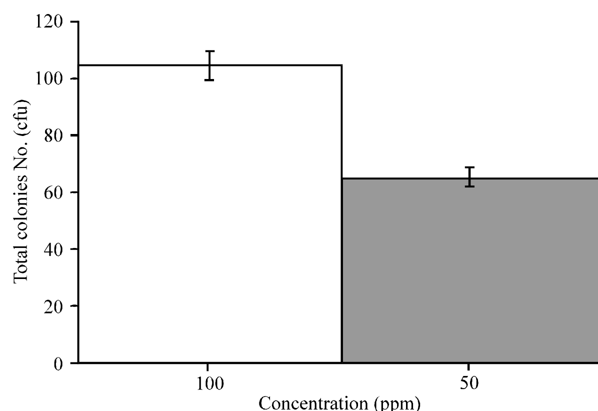

Fig. 6: No. of viable colonies of yeast cells on 100 and 50 ppm flavonoid extract

The cell shrinkage appearance could be due to damaged mitochondria (Munoz *et al*., 2012). That damage would activate proteins involved in the regulation of cell death (caspases) (Pereira *et al*., 2008). These shrinkage cells were also likely to be triggered by the collapsed of cytoskeletal proteins, such as fodolin and actin, resulted from increased caspase enzyme activity during apoptosis (Tan and Wang, 1998; Huppertz *et al*., 1999).

Number of viable yeast cells: Incubation of cells with 4% glucose for 24 h decreased the number of viable cells by 44.01% as compared to the control cells (Fig. 5). This observation suggested that beside its effect to induce petite colony, 4% glucose also produced growth inhibitory effect as shown by the reduced cell viability. The addition of flavonoid (100 ppm) also produced similar results with greater reduction (65.85%) in the number of viable cells (Fig. 6). It means that flavonoid can inhibit yeast cell proliferation. Other research showed that flavonoid can inhibit cell growth and induced apoptosis in B16 melanoma 4A5 cells (Iwashita *et al*., 2000). The catechin flavonoid reduces proliferation and induces apoptosis of murine lymphoma cells LB02 through modulation of antiapoptotic proteins (Papademetrio *et al*., 2013).

CONCLUSION

This research suggested that the flavonoid from *Guazuma ulmifolia* Lamk. possess active compound(s) which may induce apoptotic events on yeast cells. The antioxidant activity of the flavonoid extracts positively correlated with their ability to induce petite colonies. The present report is the first study to demonstrate that flavonoid extract from *Guazuma*'s leaves have inhibitory effect on cell proliferation, indicating the effect of flavonoid on other mechanisms in cellular activities.

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