ISBN 978-93-86878-21-2
15th PHNOM PENH Int'l Conference on Food, Environmental, Biological & Medical Sciences (PFEBM-18)
Aug. 8-10, 2018 Phnom Penh (Cambodia)

Antioxidant Activity of Ethanol Extract from Guazuma ulmifolia Lamk. Leaves in Modulating Apoptosis of Yeast Cells (*Saccharomyces cerevisiae*)

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***Abstract*:** *Guazuma ulmifolia L. has been used as a traditional medicine for cardiovascular diseases. Recent studies have demonstrated that antioxidant-based therapies were beneficial for treating the cardiovascular disease. In previous research, Guazuma ulmifolia L. leaves were reported to have high antioxidant activity and exhibit apoptosis induction. There were no studies concerning the effects of crude and polyphenol extract of Guazuma Ulmifolia L. leaves in modulating apoptosis, as well as explaining associated genes in apoptosis. This present work aimed to investigate the apoptosis modulation of yeast cells (Saccharomyces cerevisiae) induced by antioxidant activity and observe expression of genes responsible for yeast metacaspase (YCA1). The results showed that the antioxidant activity of crude extracts and polyphenols (100 ppm and 150 ppm) was higher than that of α-tocopherol (200 ppm) as positive control. The polyphenol extract from Guazuma ulmifolia L. leaves (150 ppm) could induce apoptosis in yeast petite cells up to 98.38%, which was almost similar to that obtained by positive control (glucose 4%), i.e. 99.51%. The use of polyphenol extract at 50 ppm increased the expression of YCA1 gene, being 3.7 times higher than that obtained by normal control. Based on our results, the extract of Guazuma Ulmifolia L. leaves served as apoptosis inducer through expressing YCA1 gene.*

Keywords: *Antioxidants, Polyphenol, Apoptosis, Petite cell, metacaspase (YCA1), Guazuma Ulmifolia L.*

1. Introduction

Apoptosis may serve a pivotal role in the pathogenesis of cardiovascular diseases. It defines as programmed cell death that can be modulated by death receptors in plasma membrane, mitochondria, and endoplasmic reticulum [1]. It is well known that apoptosis can be promoted by oxidative stress. The oxidative stress refers to a disturbance in the balance between the production of free radicals, such as reactive oxygen species (ROS), and antioxidant defense [2]. Antioxidant may modulate (either inhibit or induce) apoptosis in macrophage cells [3]. Leaves from Guazuma ulmifolia L. have been proposed to have anti-adipogenic activity capable of alleviating lipid oxidation and formation of foam cells [4]. Modulation by crude extract and flavonoid of Guazuma ulmifolia L leaves was reported through inducing petite cells or cell shrinkage [5,6].

Yeast (Saccharomyces cerevisiae) cells were often used as model in investigating the apoptotic cell death due to similar pathway between non-mammalian cells (yeast) and mammalian cells [7,8]. In mammalian cells, apoptosis occurred as consequence of protease activity, i.e. caspase. Yeast cells contained a gene (YCA1) that encodes of metacaspase type 1, a homologue of the mammalian caspase [9]. Currently, numerous studies have been made concerning the role of YCA1 as inducer of apoptotic death in yeast cells. The apoptotic modulation by YCA1 was a result of oxidative stress [7,10]; thus, this present work aimed to confirm the potential link between YCA1 and yeast apoptotic death modulated by antioxidant from crude extract and polyphenol extract of guazuma leaves.

2. Materials and Methods

2.1. Extraction of guazuma leaves and polyphenol compounds

Guazuma leaf simplisia (obtained from Research Center for Biopharmaca, Bogor) was macerated in ethanol 96% with a ratio of 1:10 (w/v) and then incubated at room temperature in a dark room for 24 h. The mixture was filtered using filter paper, while the dreg was macerated again for 24 h. Extraction was performed for 3 × 24 h. The filtrate was concentrated to produce 96% ethanol filtrate [11]. Subsequently, polyphenol extraction was performed according to previous procedure [12]. In short, a total of 2 g filtrate from crude extraction was partitioned using n-hexane, resulting in n-hexane fraction and residue. The residue (ethanolic fraction) was concentrated using rotary evaporator and fractionated using chloroform to obtain polyphenol extract.

2.2. Determination of antioxidant activity and malondialdehyde (MDA) using TBA assay

Standard curve of 1,1,3,3-tetramethoxypropane (TMP) was made according to method of Yagi [13]. TMP was diluted in 50% ethanol to produce concentration levels of 25, 50, 100, 150 and 200 ppm. The sample mixture was made by mixing sample solution (1 ml) with 2 ml of phosphate buffer (0.1 M, pH 7) and 2 ml of linoleic acid 50 mM (Sigma-Aldrich GmbH, Steinheim, Jerman) in 99.8% ethanol. Blank was also made by substituting 1 ml of sample solution with 1 ml of distilled water. Control mixture was prepared by mixing 2 ml of buffer phosphate (0.1 M, pH 7), 2 ml of linoleic acid 50 mM in 99.8% ethanol containing 200 ppm of α-tocoferol (Sigma-Aldrich GmbH, Steinheim, Germany), and 1 ml of deionized water. The mixture was incubated in inkubator at 40 oC for 8 days. Each sample (1 ml) was added with 2 ml of TCA 20% (w/v), 2 ml of TBA 1% (w/v) in acetic acid 50% and kept at 100 oC for 10 min. The mixture was cooled and centrifuged (Hettich Zentrifugen D-7200 Tuttlingen 1200, Germany) at 3000 rpm for 15 min. Absorbance measurement was carried out using spectrophotometer (Thermo Electron Corporation Genesys 10 UV, USA) at 532 nm [14].

$$\%\ inhibition\ = \frac{[\mathrm{MDA}]\text{negative control} - [\mathrm{MDA}]\ sample}{[MDA]\ negative\ control} X\ 100\%$$

2.3. Assessment of yeast apoptotic induction by guazuma leaf extracts

In this experiment, apoptosis in yeast cells and petite cell frequency were assessed. Yeast cells (BY4741 from Rika indri Astuti in Department of Biology, IPB) were subcultured in a liquid YEPD medium and harvested at stationary phase after 24 h of treatment. A total of 20 µl crude extract (50 ppm) and polyphenol extract (150 ppm) were transferred into Eppendorf containing yeast culture (100 µl) and YEPD medium (500 µl) and added with sterilized distilled water to reach total volume of 1000 µl. Yeast culture (100 µl) and YEPD medium (900 µl) were used as normal control, while positive control was made by incorporating glucose 4% in 100 µl of yeast culture, 500 µl of YEPD medium and sterilized distilled water to reach total volume of 1000 µl. All mixtures were homogenized and incubated 37 oC for 24 h [15]. After determination of petite cells, the induced yeast cells were diluted to 10-6 in liquid YEPD medium, and 100 µl of aliquots were spread onto petite YEPD medium (with less glucose level 0.1%) and added with 2% ethanol (v/v), except for normal control. The duplo experiment was performed in this section. After incubation at 28 °C for 24 h, petite cell frequency was determined as follows:

$$petite\ cells\ frequencies = \frac{\sum petite\ colony}{\sum petite\ colony + \sum normal\ colony} \times 100\%$$

2.4. Mitochondrial Damage Test

After 24-h induction, yeast BY4741 cells (1 ml) were centrifuged. Pellet was resuspended using buffer phosphate. Cell suspension was reacted with rhodamine B 100 nM for 30 in at 30 oC. Mitochondrial activities were observed under fluorescence microscope [16].

2.5. Real Time-PCR (qPCR) Experiment

The mRNA in the induced samples was extracted using RNeasy ® mini Kit (Qiagen,USA). Subsequently, cDNA was synthesized as template from mRNA using *iScript™ cDNA Synthesis* Kit (Bio-Rad, Bio-Rad Laboratories, USA), while targeted gene sequencing was *YCA1 forward* "TGGTCAGGGTTCCCACTAGG", *YCA1 reverse* "ATTGGGTTGCGCATCCTTGA" and *ACT1 forward* "GGTGTTACTCACGTCGTTCCA", *ACT1 reverse* "CAGTCAAATCTCTACCGGCCA". Gene amplification was analyzed using SYBR Green. A total of 20 µl reaction mixture was composed of 2 µl cDNA, 0.4 µl primer, 1 µl KAPA SYBR® FAST qPCR Kit *master mix* (Toyobo, Japan) and nuclease free water added to reach total volume of 20 µl. The condition for StepOne Plus Real-Time PCR (Applied Biosystem) was carried out in 40 cycles of 1 min at 95 °C (predenaturation), 0.15 min at 95 °C (denaturation). Amplification of YCA1 was determined as relative over control using CT ($2^{-\Delta\Delta CT}$) [17]. Comparison of CT ($2^{-\Delta\Delta CT}$) was determined as follows:

$\Delta\Delta CT$ = [(CT *YCA1* - CT *ACT1* without extract) – (CT *YCA1* - CT *ACT1* treated with extract)].

2.6. Statistical Analysis

Experimental design was made according to completely randomized design, while data were statistically evaluated using one-way analysis of variance (one-way ANOVA) in Minitab 16. Significant difference between means was compared using Tukey test at $p<0.05$.

3. Results and Discussion

3.1. Antioxidant Activity of Guazuma Leaves

Antioxidative effect of the extracts was evaluated according to formation of MDA. Addition of vitamin E (positive control) was able to attenuate MDA level up to 90.13%. Furthermore, the results exhibited that treatment with crude extract and polyphenol extract of guazuma leaves at 100 ppm demonstrated the highest antioxidant effect on reducing MDA level, i.e. 95.43% and 92.05%, respectively. The value appeared to be slightly different compared to that obtained by both extracts at 150 ppm (91.45%). However, we found that antioxidant activity of the extracts was higher than that of vitamin E, as depicted in Figure 1.

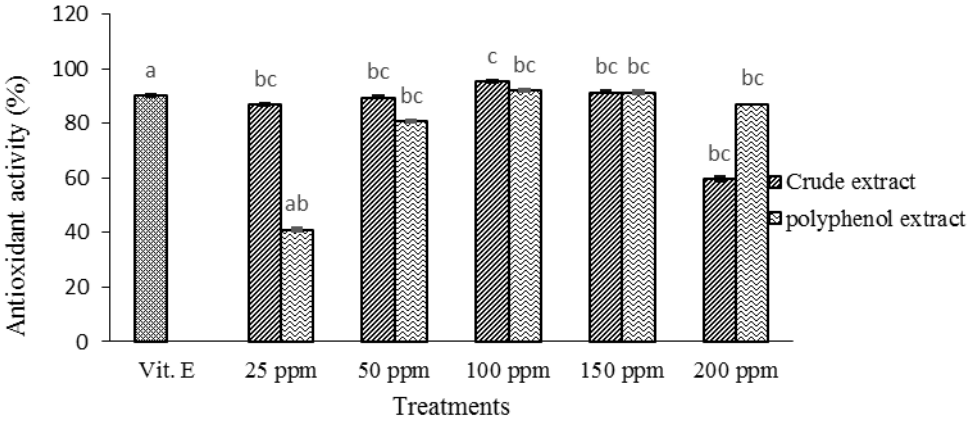

Fig 1 Antioxidative effects of vitamin E, crude extract and polyphenol extract from guazuma leaves. Similar characters above the bar chart showed insignificant difference ($p>0.05$).

The result was in accordance with that reported by Syaefudin [6], finding that flavonoid extract of guazuma leaves at 100 ppm could demonstrate a higher antioxidant activity in comparison with vitamin E. Presence of secondary metabolites in plants is responsible for antioxidative feature. In this regard, polyphenol is commonly used as radical scavenger. Hidayat [18] reported that ethanol extract of guazuma leaves contained some compounds such as phenolic, flavonoid NaOH, triterpenoids, quinones and tannins. In guazuma leaves, the potential compounds associated with antioxidant and anti-triglyceride activity included flavonoid, tannins,

carotenoid and phenolic acids [19]. Batubara [20] successfully fractionated quercetin in guazuma leaf extracts which could reduce cholesterol level.

3.2. Kemampuan Yeast Apoptosis Induction by Guazuma Leaf Extracts

Apoptosis induction. Apoptosis in yeast cells was modulated by administration of guazuma leaf extracts. The emergence of apoptotic activity could be seen from colony formation, in which apoptosis was indicated by formation of petite cells in comparison with normal cells [15]. The results showed that yeast cells after induction with glucose 4% underwent cell shrinkage. As previously reported by Lusiana [5], Syaefudin [6] and Sriwijayanti [21], glucose 4% could successfully promote generation of petite cells. Additionally, crude extract and polyphenol extract of guazuma leaves also exerted similar response on formation of petite cells. Extract polyphenols have a higher number of petite cells than induced crude extracts (Figure 2).

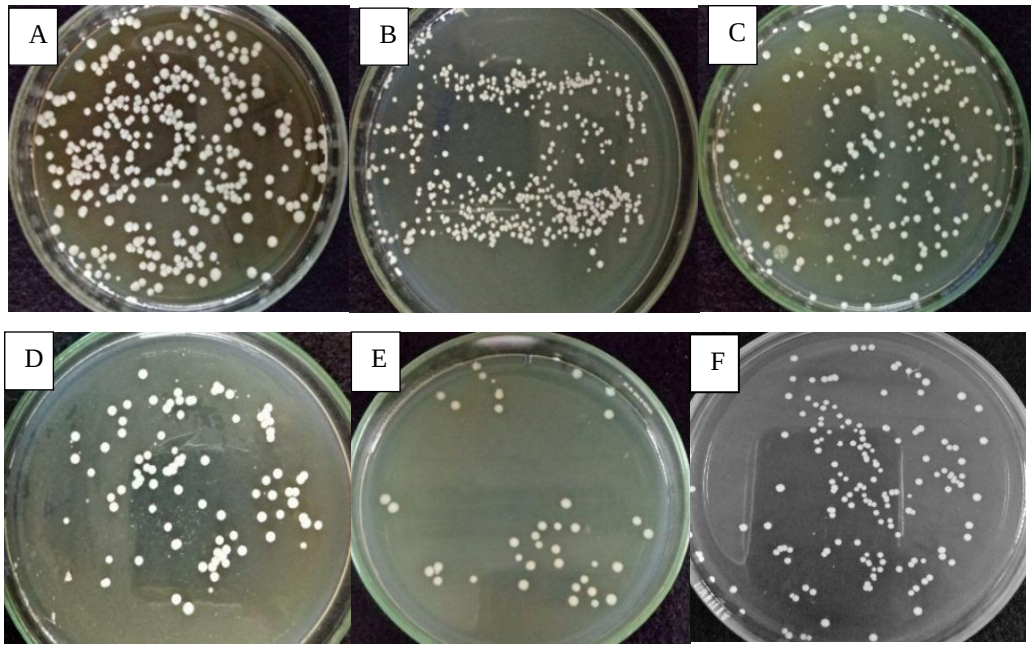

Fig.2 Appearance of normal yeast colony (A) and treated yeast colony with glucose 4% (B), ethanol 1% (C), crude extract of guazuma leaves at 50 ppm, (D) crude extract of guazuma leaves at 150 ppm (E), and polyphenol extract of guazuma leaves at 50 ppm (F).

Petite cell frequency. After induced with guazuma leaf extracts, petite cell frequency was calculated by counting number of petite colony and normal colony. As presented in Table 1, colony treated with distilled water showed a low petite frequency (0.7%), but it increased up to 99.51% with induction of 4% glucose. Petite frequency induced by 4% glucose was almost comparable with that induced by crude extract and polyphenol extract at 50 and 150 ppm (Table 1). Syaefudin [6] asserted that flavonoid extract of guazuma leaves had less effects on formation petite cells than that of 4% glucose. This discrepancy resulted from difference in solvents for extraction. In our experiment, induction by control treatment (1% ethanol) was performed to discover its effect on cell shrinkage. As a result, the application of 1% ethanol caused much lower percentage of petite cells (20.88%) compared to extract treatments, suggesting that it could be less effective as apoptotic inducer.

Table I Frequency of petite cells after induced with various concentrations of crude extract (CE) and polyphenol extract (PE).

Treatments	Petite colony	Normal colony	Petite frequency (%)
Normal	2	282	0.70±0.228[d]
1% Ethanol	40	151	20.88±2.63[c]
4% Glucose	1	205	99.51±0.14[a]
CE 50 ppm	5	41	88.29±1.26[b]
CE 150 ppm	3	80	96.38±3.61[ab]
PE 50 ppm	7	297	97.69±3.76[a]
PE 150 ppm	3	191	98.38±0.19[ab]

Note: Dissimilar superscripts following the number in same column showed significant difference ($p<0.05$).

Metabolic activity responsible for yeast petite cells was influenced by incubation time, medium composition, and induction by high concentration of glucose [21]. With 0.5% glucose and 2% ethanol (medium for petite), normal cells were capable of utilizing ethanol as energy source, while petite cells could not.

3.3. Mitochondrial Damages

Selain In addition to formation of petite cells, apoptotic cells also suffered severe alterations of cytoplasmic organelles and disfunction of mitochondria [21,22]. Increased caspase activity during apoptosis was also responsible for disruption of cytoskeleton membrane [22]. Mitochondrial damages activated protein (caspase) that regulate cell death [23]. Mitochondrial changes observed with Rhodamine B staining were depicted in Figure 3. Normal cells and cells treated with 1% ethanol emitted red fluorescence, while no fluorescence was found at cells treated by 4% glucose and extracts of guazuma leaves. The fluorescence by yeast cells occurs as response to presence of mitochondrial activities. In contrast, the apoptotic cells could not display it due to disrupted mitochondrial function.

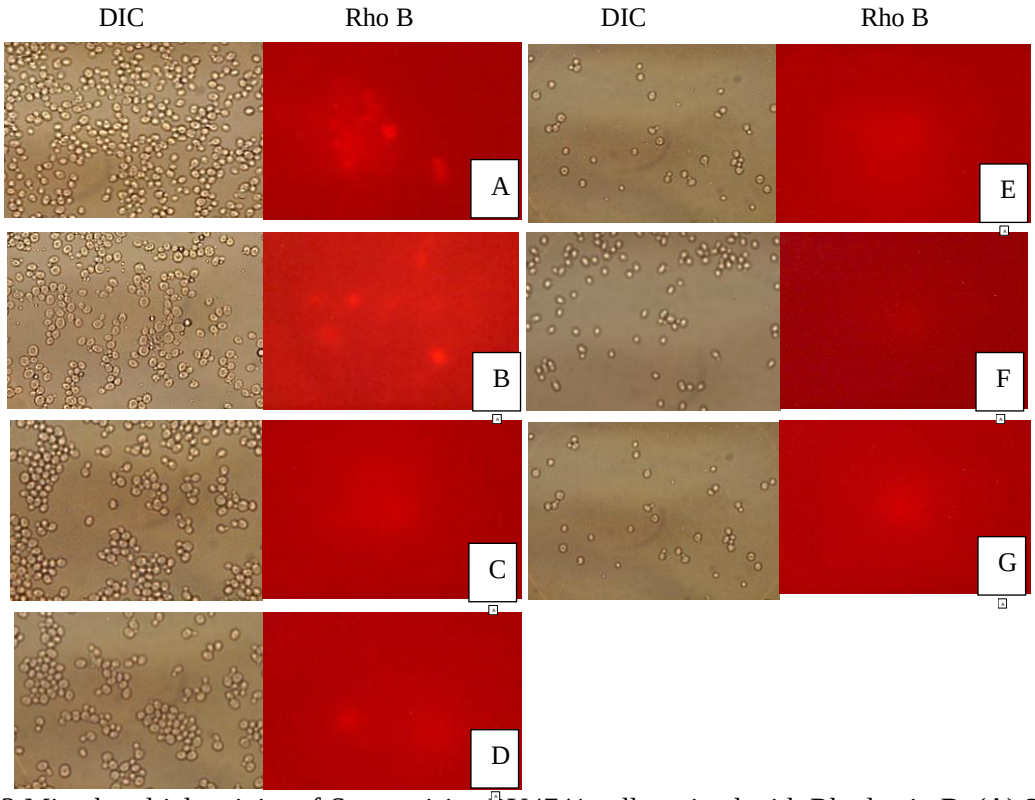

Fig 3 Mitochondrial activity of S. cerevisiae BY4741 cells stained with Rhodamin B. (A) 2% YPD, (B) 1% ethanol, (C) 4% glucose, (D) CE 50 ppm, (E) CE 150 ppm,(D) PE 50 ppm, (E) PE 150 ppm

The mitochondrial disfunction was caused by fragmentation, accumulation of ROS, and changes in membrane potential due to induction of 4% glucose and exposure to guazuma leaf extracts. Glucose enrichment without adding other nutrients would promote ROS accumulation in mitochondria, leading to apoptosis and formation of petite cells [15]. Under hyperosmotic stress (high level of glucose or sorbitol), mitochondria underwent swelling activity [24]. Brown [25] reported that high concentration of glucose would provoke changes in structural and functional properties of S. cerevisia cells including respiration rate, and activity of NADH oxidase and cytochrome c.

3.4. Expression of YCA1 gene

Based on Real-Time PCR experiment, expression of YCA1 gene in positive control (4% glucose treatment) was higher, being 2.9 times higher than normal control. Meanwhile, cells treated with guazuma leaf extracts (PE 50 ppm) showed the highest level of YCA1 gene expression, i.e. 3.7 times higher than normal control and 0.8 times higher than positive control. Other treatments resulted in variation of YCA1 gene expression either higher than normal control or lower than positive control (Figure 4). Expression of YCA1 gene could promote cell death (apoptosis) [7, 9]. In this regard, we emphasized that cell apoptosis through YCA1 gene expression could be achieved by induction of polyphenol extracts from guazuma leaves. Similarly, this apoptotic mechanism by YCA1 gene expression was also present in cells after induced by hydrogen peroxide, acetic acid, stres hyperosmotic, arsenic compounds, caffeine, and metal ions [9]. Programmed cell death induced by acetic acid (AA-PCD) was indicated by the YCA1 gene expression causing the increased apoptotic activity, while Δyca1 treatment enabled to reduce cell death [26].

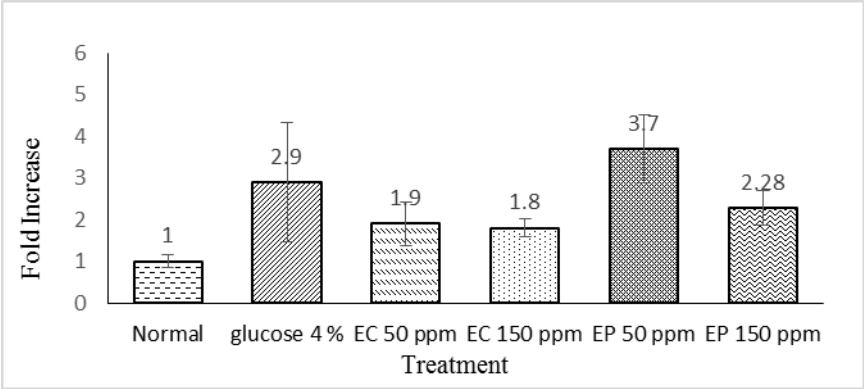

Fig. 4 Variation in YCA1 gene expression after normalized with ACT housekeeping gene using 2-ΔΔCT quantification method

4. Conclusion

The high antioxidant activity of crude extract and polyphenol extract from guazuma leaves demonstrated appreciable results in apoptosis induction. The apoptotic activity was recognized by formation of petite cells, damaged mitochondria, and high level of YCA1 gene expression.

5. Acknowledgement

Authors would like thank to Lembaga Pengelola Dana Pendidkan (LPDP) Indonesia for providing research grant awarded to the first author.

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