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Original Research Article

Anticancer activity of okra raw polysaccharides extracts against human liver cancer cells

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Abstract

Purpose: *To examine raw polysaccharides extract of okra (Abelmoschus esculentus L.) as an anticancer agent against human liver cancer cells.*
Methods: *Okra raw polysaccharide extract (ORPE) was obtained by ethanol extraction from the raw fruit. Huh7it cells were grown in DMEM (Dulbecco's Modified Eagle Medium) and cultured for 24 h prior to treatment with the extract. The cell culture was divided into 3 groups, viz, negative control group (KN), positive control group (KP, treated with 10 µg/mL doxorubicin), and ORPE (P) group. ORPE group was divided into 5 subgroups based on the dose used for treatment, viz, 50 (P1), 100 (P2), 200 (P3), 400 (P4), and 600 µg/mL (P5). Huh7it cell proliferation was measured by MTT assay. while measurement of Huh7it cell apoptosis, necrosis, and cell cycle analysis were carried out using Annexin V FITC-PI antibody test and flow cytometry.*
Results: *ORPE significantly inhibited Huh7it cell proliferation and induced apoptosis. ORPE treatment with 600 µg/mL extract caused 5.82 % late cell apoptosis and 5.62 % early apoptosis. Cell cycle arrest occurred during G0/G1 phase.*
Conclusion: *ORPE is a potential anticancer agent against human liver cancer cells due to its ability to induce apoptosis of huh7it cells by promoting cell cycle arrest during G0/G1 phase.*

Keywords: *Abelmoschus esculentus L., Anticancer, Apoptosis, Cell cycle, Cell proliferation, Liver cancer, Raw okra polysaccharides*

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INTRODUCTION

Cancer is one of the top listed death-causing diseases in the world. The World Health Organization reported that the two most common types of cancer in the world are lung cancer (1.69 million victims) and liver cancer (788,000 victims) [1]. Liver cancer or hepatocellular carcinoma (HCC) is an uncontrolled growth of malignant cells in the liver that caused by abnormal cells in the liver (primary) and other body parts (secondary). Surgery and

chemotherapy are the main therapeutic methods to treat HCC patients. Also, as a prevention effort, Hepatitis B immunization can be used. However, the application of therapeutic drugs has several limitations, such as hemopoietic suppression, limited efficacy, immunotoxicity and drug resistance [2].

Polysaccharides have long been recognized as the anticancer agent with low toxicity and slight of side effects [2-4]. Recently, there is a shift of interest from microbial polysaccharides to plant polysaccharides due to the lesser of toxicity and side effects from the latter [5]. Plant polysaccharides exerted anti-cancerous activities, either by enhancing the immune system [6] or by causing apoptosis through the caspase pathways [2,3].

Okra (*Abelmoschus esculentus* L.), a flowering plant from Malvaceae family that is found in tropical, subtropical and warm temperate regions around the world [7,8]. The pods are rich in polysaccharides, vitamin C, secondary metabolites, minerals, and fiber, but low in calories [9]. Besides being consumed as food, okra is also used as traditional medicine to treat dysentery and diarrhea [10]. Flavonoid compounds such as oligomers catechins, quercetin, and vitamin C are antioxidants. *Okra's* phenolic component such as tannins could act as antibacterial agent by damaging the bacterial cell membrane and inhibiting their enzymes activities [11]. Recently, Wahyuningsih *et al* found that okra polysaccharides acted as immune-modulators by enhancing B-lymphocytes proliferation and spleen weight through the activation of NFκB transcription factor [12].

The anticancer effect of okra on different types of cancer cells had been revealed. Lectin, isolated from okra was able to induce significant cell growth inhibition in human breast cancer (MCF7) cells up to 63% [13]. Hot buffer extraction of okra pod could induce apoptosis in melanoma cells by interacting with Gal-3 and cause a cell cycle arrest in G2/M phase [14]. Moreover, the fraction of okra polysaccharide extract had inhibition effect on MCF-7, Hela, and MCG803 cells with the lowest survival rates of 63.90, 63.5,1 and 67.71 %, respectively [15].

However, there are no studies that have addressed the anticancer potential of raw polysaccharides from okra pods grown in Indonesia for the treatment of human liver cancer. Hence, this study was design to evaluate the anticancer activity of okra's raw polysaccharides extract (ORPE) *in vitro*.

EXPERIMENTAL

Preparation of polysaccharides

The okra fruits were obtained from traditional markets in Surabaya, Indonesia. Fresh okra pods (500g) were rinsed with water, then cut and macerated with 500 ml of distilled water overnight. Supernatants were collected and centrifuged at 4300 rpm for 5 min, followed by precipitation with absolute ethanol (1:1 ratio) and incubated at 4 ^{o}C, 24 hour then centrifuged. The pellet was dissolved in distilled water and centrifuged. The supernatant was collected, lyophilized, and labelled as okra raw polysaccharide extract (ORPE) [9]. The stock solution was made by solubilizing ORPE powder (5 mg) into DMSO (50 µL).

Tumor cell lines and culture

Human liver cancer cell line Huh7it was grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 µg/mL) as a monolayer at 37 ^{o}C in a humidified CO_2 (5 %) incubator to promote the exponential proliferation. The cells were divided into 4 groups, i.e., Splenocyte control group (K), Huh7it control group (KN), positive control group that given with 10 µg/mL doxorubicin (KP), and ORPE treatment group (P). The ORPE treatment group were divided into 5 sub-groups based on their dose, i.e., 50 (P1), 100 (P2), 200 (P3), 400 (P4), and 600 µg/mL (P5).

Cell growth inhibition assays

In vitro cell proliferation was measured using the MTT [3-4, 5-dimethylthiazole-2-il) -2,5 diphenyltetrazolium bromide] assay as described by Hussain *et al* with slight modification [16]. Cells from the CO_2 incubator were observed for their sterility, dispensed in 96-multiwell plates at a density of 5×10^3 cells per well and pre-cultured for 24 h. Then, cells were treated with ORPE at various final concentrations (50, 100, 200, 400, and 600 µg/mL). After 48 h of incubation, 15 µL of MTT solution (0.5 mg/mL) in 135 µL DMEM was added to each well. The plates were incubated at 37 ^{0}C for an additional 4 h until blue formazan crystals were formed. Then, the supernatant was aspirated and cells were suspended into 50 µL of DMSO. The absorbance was measured using microplate reader (GloMax®-Multi+, Promega Corp., USA) at wavelengths of 560 and 750 nm. Percent surviving cells (cell growth, G) was calculated as in Eq 1.

$G\ (\%) = (A_{560} - A_{750})/(C_{560} - C_{750})$ ……. (1)

where A and C are the absorbance of test and control wells, respectively. x 100. The results represent the mean of three independent experiments.

Determination of apoptosis and necrosis

The apoptotic effect of ORPE on Huh7it cells was determined with Annexin V-FITC staining assay using a flow cytometer. As described earlier, the Huh7it cells ($1x10^5$ cells per well) were treated with five concentrations of ORPE (50, 100, 200, 400, and 600 µg/mL) and cultured for 48 h. Then, cells were harvested by trypsinization, rinsed with cold PBS, and transferred into microtubes. After centrifugation (4◦C, 2500 rpm) for 5 min, cells were washed two times with 200 µL Cell Staining Buffer (BioLegend, USA) then resuspended in Annexin V Binding Buffer (BioLegend, USA) at a concentration of 0.25 - 1.0 x 10^7 cells/mL. Five microliter of FITC Annexin V (BioLegend, USA) and 10 µL of propidium iodide solution (BioLegend, USA) were added to each microtube, vortexed and incubated for 15 min at room temperature (25 °C) in the dark. Annexin V binding buffer (400 µL) was added to each tube. The Annexin-V and PI fluorescent intensities were analyzed using flow cytometry (BD FACSCalibur System, San Jose, CA, USA).

Flow cytometry

The Huh7it cells (1 x 10^5 cells per well) were treated with five concentrations of ORPE (50, 100, 200, 400, and 600 µg/mL) and cultured for 48 h. Cells were harvested by trypsinization, washed by cold PBS and transferred into microtubes. The cells were fixed with cold 70 % ethanol and incubated for 30 min at 4 °C, centrifugated at 2000 rpm and re-suspended in 1xPBS. The centrifugation and resuspension were repeated. After discarding the supernatant, RNase 100µg/mL was added to each microtube, followed by the addition of 425 µL Cell Staining Buffer (BioLegend, USA) and 25 µL of propidium iodide solution (BioLegend, USA). The stained cells were analyzed using a flow cytometer (BD FACS Calibur System, San Jose, CA, USA).

Data analysis

Cell apoptosis, necrosis, and cycle were analyzed using BD Cell Quest™ Pro software. Cell viability, apoptosis, necrosis, and NKC activity data were analyzed by statistical analysis using SPSS 21 software for Windows, and one-way ANOVA followed by Duncan test. The results are presented as mean ± SD with $p \leq 0.05$ considered statistically significant.

RESULTS

The effect of ORPE on cancer cell growth was determined by treating the human liver cancer cell (Huh7it) with various doses of ORPE for 48 h. The data of cell proliferation can be seen in Figure 1. The results of growth inhibitory assay showed that ORPE at a dose of 600µg/mL was the only one group that has significant difference from the normal control group (KN). The positive control group that treated with doxorubicin showed the lowest cell proliferation among others. This result showed that the ORPE at 600 µg/mL dose had an inhibitory effect on Huh7it cells with the lowest survival rates of 84.85 %.

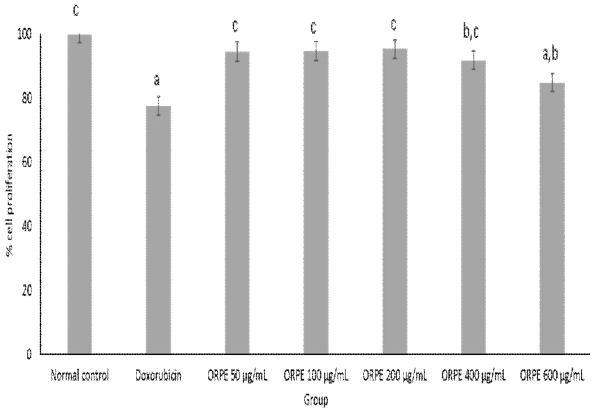

Figure 1: Huh7it cell growth after ORPE treatment for 48 hours. The different letters indicated significant difference at $p \leq 0.05$

To determine the effect of ORPE on cell apoptosis and necrosis, cells were treated with Annexin-V FITC and PI. The percentage of early apoptosis, late apoptosis, and necrosis were measured using flow cytometry. The data are shown in Figure2. Doxorubicin group (KP) showed the highest rate of early apoptosis (5.7 %) followed by the ORPE group at a dose of 600 µg/mL (P5) that causing 5.62 % of the cells experienced early apoptosis. While, the highest rate of late apoptosis happened at the ORPE group with a dose of 100 µg/mL. Total of 5.82% of the group cells experienced late apoptosis. The highest percentage of cells experiencing necrotic occurred on ORPE at 600 µg/mL. This result indicates that the ORPE at a dose of 600 µg/mL caused the high rates of apoptosis and necrosis of cancer cells when compared to the other groups (Figure 2 and Figure 3).

After determining the apoptosis and necrosis in each group, the phase of where the cell cycle

arrested was analyzed using the flow cytometry (Table 1). The result showed that all groups experienced peak rate on G0/G1 phase and decreased on S phase. This indicates that most of the cells were arrested on G0/G1 phase. P1, P3, and P4 showed the highest number of cell in G0/G1 phase compared to other groups.

DISCUSSION

Cancer cell death occurs through two pathways: extrinsic pathway or death receptor pathway (DR) and intrinsic pathway or mitochondrial pathway [17]. One of the extrinsic pathways is performed by NK cells which are part of the innate response included in the lymphocyte class [17,18]. While mitochondrial pathway involves various groups of proteins such as Bax, Bcl, p53and caspase group which further can cause DNA damage and apoptosis in cancer cells [17,18].

This study found that the ORPE has the ability to reduce the metabolism of cancer cells. It also has potential as an anti-cancer agent by inhibiting the cell growth up to 15 % at the highest dose.

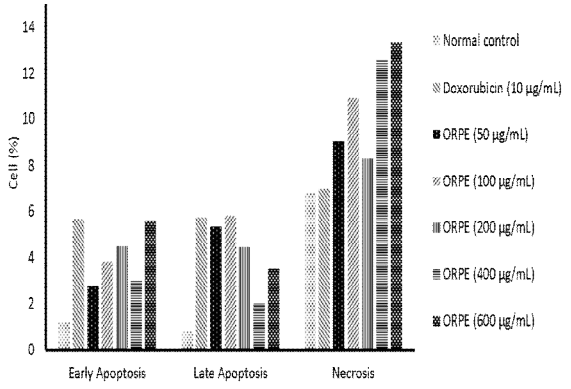

Figure 2: Percent early apoptosis, late apoptosis, and necrosis of human liver cancer cell Huh7it from each group

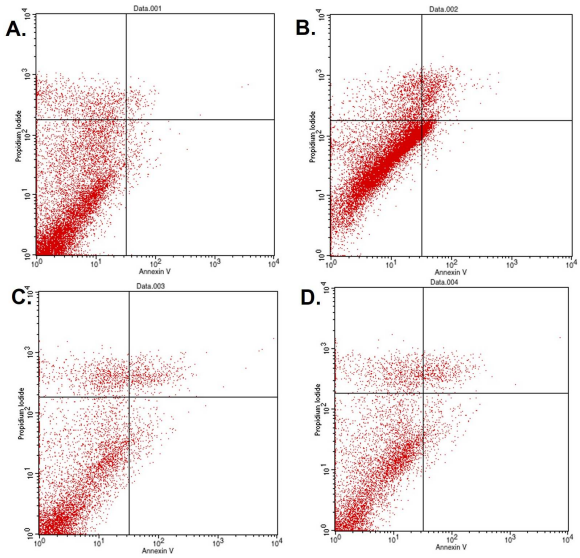

Figure 3: Huh7it cell analysis using flow cytometry after cells were stained with annexin V-FITC and PI. Upper left: necrosis, upper right: late apoptosis, bottom right: early apoptosis, bottom right: living cell. A: normal control, B: positive control (doxorubicin 10 µg/mL), C: ORPE 50 µg/mL, D: ORPE 100 µg/mL

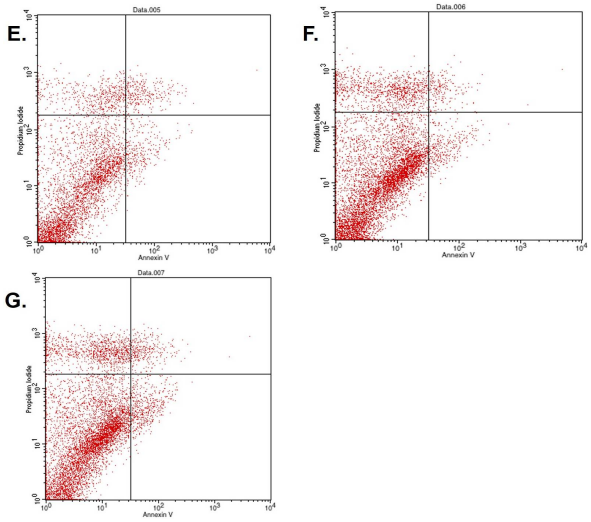

Figure 4: Huh7it cell analysis using flow cytometry after cells were stained with annexin V-FITC and PI. Upper left: necrosis, upper right: late apoptosis, bottom right: early apoptosis, bottom right: living cell, E: ORPE 200 µg/mL, F: ORPE 400 µg/mL, G: ORPE 600 µg/mL

Table 1: Percent G0/G1, S, and G2/M phases of Huh7it cell cycle

Group	G0/G1 (%)	S (%)	G2/M (%)
Normal control	19.93	7.06	2.74
Positive control	20.62	9.51	2.38
ORPE (50 µg/mL)	30.13	10.79	4.63
ORPE (100 µg/mL)	21.75	7.05	2.75
ORPE (200 µg/mL)	28.54	9.37	3.49
ORPE (400 µg/mL)	23.53	8.79	3.65
ORPE (600 µg/mL)	25.74	10.02	4.73

Karnjanapratum and You reported that the raw polysaccharide extract from *Monostroma nitidum* with rhamnose content of 51 % is able to reduce the growth of HeLa cancer cells by 30 % at a concentration of 500 µg/mL [19]. Whereas the ORPE containing 29.9 % rhamnose [9]. However, ORPE used in this study is containing some other substances such as galacturonic acid and glucuronic acid. Therefore, the anticancer effect is not yet proven to be rhamnose-related only.

Cancer growth inhibition could lead to apoptosis, that is cell death programmed from the cells. Flow cytometry data showed that the highest dose of 600 µg/mL was able to cause the cell to experience the highest early apoptosis phase. Whereas, the lowest dose of ORPE 50 µg/mL was able to cause the cell to experience the highest late apoptosis phase. A balance of early and late apoptosis occurred in the treatment of dose 200 µg/mL indicates that cells reacted differently to each dose. ORPE at a dose of 600 µg/mL could react on cancer cells mostly in the early apoptosis. It is conceivable that the higher ORPE could help to enhance the initiation of apoptosis in Huh7it cancer cells. Therefore, it could decrease cell growth. At low doses, with low levels of polysaccharides, the cells percentage was highest in the final phase of apoptosis. However, the high number of apoptotic cells in the ORPE treatment group compared to normal group indicates that the caspase protein was being activated.

This result is supported by the findings of Zhang *et al* that the administration of *Grifola frondosa* polysaccharides at dose of 50 µg/mL in MCF-7 breast cancer cells is able to increase the activation of caspase-8 and caspase-3 and cause 21% of MCF-7 cells experienced apoptosis [3]. On the mitochondrial pathway, apoptosis is caused by the release of cytochrome-c through the formation of channels associated by mitochondrial permeability transition pore (PTP) and Bax protein. Cytochrome-c that released into the cytosol will form an apoptosome complex along with Apaf-1, ATP, and caspase 9 [3]. This apoptosome will activate caspase-3 which plays a role in cutting the cytoskeleton and cleavage of gelsolin, a protein involved in maintaining cell morphology, until cells undergo apoptosis.

Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, condensation of chromatin and cytoplasm) which are well measurable by flow cytometry. There is a special DNA peak appeared when cells experienced apoptosis. This peak is thought to be one of the characteristics of apoptosis and usually called the sub-G0/G1 peak or apoptotic peak. This apoptotic process was also related to role of p53 which plays a critical role in p21 induction following DNA damage [20]. Interestingly, this study found that treatment with ORPE for 48 h resulted in the accumulation of cells in the G0/G1 phase. Cells treated with lower concentration of ORPE drastically increased the sub-population of G0/G1 phase when compared to the control groups. Even though the accumulation of cells at each ORPE concentrations were dynamic, all groups were found to arrest cells in the G0/G1 phase.

CONCLUSION

Okra raw polysaccharides extract exerts anticancer effect on Huh7it cancer cells by significantly decreasing cells proliferation and inducing cell apoptosis. Thus, okra raw polysaccharides extract may be potentially useful for the management of liver cancer patients.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The project work was designed by Suhailah Hayaza, Win Darmanto, and Sri Puji Astuti Wahyuningsih. The experiments were carried out by Suhailah Hayaza, Sri Puji Astuti Wahyuningsih, Raden Joko Kuncoroningrat Susilo, Adita Ayu Permanasari, and Saikhu Akhmad Husen. The manuscript preparation and editing were carried out by Suhailah Hayaza, Dwi Winarni, Hunsa Punnapayak, and Win Darmanto.

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