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FULL PAPER

Extract of ceplukan (*Physalis angulata* L.) inhibited proliferation and induced apoptosis in myeloma cell line

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Abstract

Cancer is one of the main causes of death globally. Thus, research to develop a new cancer drug is still ongoing. *Physallis angulata* L. (ceplukan) is one of the Indonesian plants that has been shown to display anti-cancer activity. This research aimed to examine the cytotoxic effect of ethanol extract of *P. angulata* L., the effect on cell proliferation and the apoptosis-inducing ability against myeloma cells. *P. angulata* L. plant was extracted using 96% ethanol. The observations of the cytotoxic effect of extract and the proliferation of myeloma cells were done by MTT assay. The effect of the extract on apoptosis was determined by double staining method using ethidium bromide-acridine orange. Markers for apoptosis were measured by immunocytochemistry test. The results showed that the extract of *P. angulata* exhibited cytotoxic effects on myeloma cells with an IC_{50} value of 70.92 µg/ml. The extract inhibited the proliferation of myeloma cells and induced apoptosis in myeloma cancer cells by upregulating the expression of p53 and Bax. This result suggested that ethanolic extract of *P. angulata* L. had the potential to be developed as blood cancer drug. Further study on the isolation of the bioactive compound and *in vivo* mechanism is in progress.

1. Introduction

Cancer is a disease that causes death worldwide. At present, the treatments of cancer such as surgery, radiation, and chemotherapy have not produced encouraging results (Davis *et al*., 2003). Thus, research to find more effective cancer drug with minimal side effects are being conducted. Natural products have an essential role in drug discovery and development (Zhang *et al.,* 2017). Indonesia is a country rich in plant-based natural medicine that has been used for generations by the community to treat many diseases including cancer. *Physallis angulata* L. (ceplukan) is a member of the *Solanaceae* family which is spread out in tropical countries, including Indonesia. This plant has the potential to be developed as anti-cancer medicine (Sun *et al.,* 2017). Various studies showed that this plant has antitumor, cytotoxic effect, inhibition of the ubiquitin pathway-proteasome (He *et al.,* 2013; Xia *et al.,* 2016; Yang *et al.,* 2018), immunomodulators (Sun *et al.,* 2011), antibacterial (Yang *et al.,* 2016), and anti-inflammatory (Qiu *et al.,* 2008; Sun *et al*., 2016). The chemical content of these plants that are responsible for these activities includes steroids, flavonoid glycosides, and alkaloids (Lee *et al.*, 2008; Sun *et al*., 2017).

Previous studies have shown that *P. angulata* extract exhibited immunomodulatory activities (Sun *et al*., 2011). The ethanol extract of *P. angulata* also induced cytotoxic activity against several cell lines such as HA22T (hepatoma), KB (nasopharynx), Colo 205 and Calu (human lung epithelial cancer) (Chiang *et al*., 1992). Petroleum ether fraction of *P. angulata* plant can inhibit proliferation and induce apoptosis in HeLa cells (Maryati and Sutrisna, 2011). Ethanol extract of *P. angulata* is potential as an immunomodulatory agent because it can induce the differentiation of bone marrow cells (BMC) into macrophages (da Silva *et al*., 2014). Other studies also showed that flavonoid glycosides from *P. angulata* exhibited cytotoxic effects against cancer cells (Ismail and Alam, 2001). Other compounds in *P. angulata* that have anticancer potentials are physalin and

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withanolide (Zhang *et al*., 2017). Sun *et al*., (2017) isolated five new physalin compounds from the stems and leaves of *P. angulata* and all the five compounds showed antiproliferative activity against human cancer cells (C4-2B, 22Rv1, 786-O, A-498, ACHN, and A375-S2). The latest study using a reactivity-based screening (RBS) approach managed to get five new withanolide compounds with cytotoxic activity (Zhang *et al*., 2017).

At present, the chemotherapy drugs such as etoposide (Karpinich *et al.,* 2002), vincristine (Groninger *et al.,* 2002), 5-fluorouracil (Liu *et al.,* 2008; Mhaidat *et al.,* 2014), or doxorubicin (Eom *et al.,* 2005) work by inducing apoptosis in cancer cells. Therefore, agents that can induce apoptosis or affect cell cycles attracts many researchers. Currently, there is no study report on the cytotoxic effect of this plant on myeloma cells. The aim of this study is to examine the anti-cancer potential of *P. angulata* plant against myeloma cell line.

2. Materials and methods

2.1 Plant Sample

P. angulata L. plant was collected from Gantiwarno, Klaten, Central Java, Indonesia and was identified by botanist Deny Wahyu, S.Si in the Laboratory of Morphology and Plant Physiology, Department of Biology, Faculty of Education and Teaching, University of Muhammadiyah Surakarta, Central Java, Indonesia with the voucher specimen number 122/A.E.1/LAB. BIO/III/2010.

2.2 Extraction

The whole plant of *P. angulata* L. was dried using the oven at 50ºC for 3 days. The dried plant was blended into fine powder followed by extraction with 96% ethanol. The liquid extract was dried by a rotary evaporator. The thick extract obtained was then used for cytotoxic tests, inhibition of cell proliferation, and apoptosis observation.

2.3 Cytotoxic test

Myeloma cells were obtained from Parasitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The 2.0 x 10^4 cells/well were seeded into 96-well plate and incubated at 37ºC, incubator (5% CO_2) for 24 hrs. Cells were treated with various concentration of *P. angulata* L. extract (31.25; 62.5; 125; 250; 500 µg/mL). The treated cells were incubated in an incubator (5% CO_2) for 24 hrs at 37ºC. Cells were washed with Phosphate Buffer Saline (PBS), then added with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide), incubated for 4 hrs at 37ºC. Cells were added with regent stopper (SDS reagent (10%) in HCl 0.01 M), incubated overnight at room temperature, 28ºC (Handayani *et al.,* 2017). The absorbance was measured using the ELISA reader at a wavelength of 550 nm. The percentage of viable cells was calculated from absorbance data. Furthermore, to calculate the IC_{50}, a linear regression equation between the concentration of extract and percent of viable cells was formulated.

2.4 Observation of proliferation inhibition

Observation of myeloma cell proliferation inhibition was carried out by using the MTT method, concentration samples were used below IC_{50} values. Observations were conducted at 24, 48, and 72 hrs (Cancer Chemoprevention Research Center, 2009).

2.5 Apoptosis Observation

Cells with a density of 1.5 x 10^4 cells/well were seeded on coverslips in a 24-well plate and incubated in 37ºC incubator (5% CO_2) until confluent. Then, cells were incubated with extract for 24 hrs. Next, the medium was taken, and the cells were washed using PBS. The coverslip containing cell was placed into the object glass followed by double staining using 10 mL 1X working solution acridine orange-ethidium bromide (Sigma Aldrich). It was left for 5 mins before immediately observed under a fluorescent microscope (Zeiss MC 80). Green fluorescent indicated the viable cells and orange fluorescence marked the apoptosis cells.

2.6 Immunocytochemistry assay

Cells with a density of 1.5 x 10^4 cells/well were seeded on coverslips in a 24-well plate, incubated 37ºC incubator (5% CO_2) until confluent. Then, cells were incubated with 70 mg/mL extract of *P. angulate* plant for 24 hrs. The medium was removed and washed with PBS. Cells were fixed with acetone for 10 mins and washed with PBS. Cells were added by normal mouse serum (1:50) for 15 mins, removed (without washing), incubated with monoclonal antibody anti-p53 (Dako) and anti-Bax (Dako) (1:50 dilution) for 60 mins at 28ºC, then a washed in PBS 3 times. The preparations were incubated in biotin for 10 mins and washed with PBS twice for 5 mins, followed by incubation in streptavidin-peroxidase for 10 mins and washed with PBS (2 times for 5 mins), incubated in DAB for 3-8 mins and washed with distilled water. Cells were stained with hematoxylin for 3-4 mins and washed with distilled water. Coverslips were moved into object-glass, fixed with ethanol and xylol. Protein expression was observed using a light microscope (Nikon YS 100). Compare with the cells without certain protein, cells that expressed certain proteins were brown/dark in color.

3. Results and discussion

3.1 Cytotoxic test and observation of myeloma cell inhibition

Cytotoxic tests were done using the MTT method [3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide]. Compared to dead cell, viable cells can cleave MTT to insoluble purple formazan (Doyle and Griffiths, 2000). Formazan was then dissolved using 10% SDS in 0.1 N HCl and the absorbance was measured with ELISA Reader. The absorbance of solubilized formazan was proportional to the number of viable cells (Cancer Chemoprevention Research Center, 2009). This cytotoxic test illustrated the ability of extracts to inhibit cell growth. Increasing the concentration of extract resulted in a decrease in the percent of viable cells (Figure 1). Based on calculations using linear regression analysis, *P. angulata* extract had cytotoxic activity in myeloma cells with IC_{50}value of 70.92 µg/mL. The morphology of the control cell (no treated extract) appeared to be round and clear. In contrast, the treated cell become shrink and had an irregular shape (Figure 2). Previous research showed that Physalin B, D, F compounds isolated from *P. angulata* plants displayed strong cytotoxic activity on KB cells, A431, HCT-8, PC3 and ZR751 with IC_{50} less than 4 µg/ml (Kuo *et al*., 2006). Withangulatin A and withangulatin I which are with the anolide compounds isolated from *P. angulata* plants have been shown to have cytotoxic effects on colorectal carcinoma (COLO 205) and gastric carcinoma (AGS) with IC_{50} from withangulatin A are 16.6 and 1.8 µm, and IC_{50} from withangulatin I, are 53.6 and 65.4 µm, respectively (Lee *et al*., 2008).

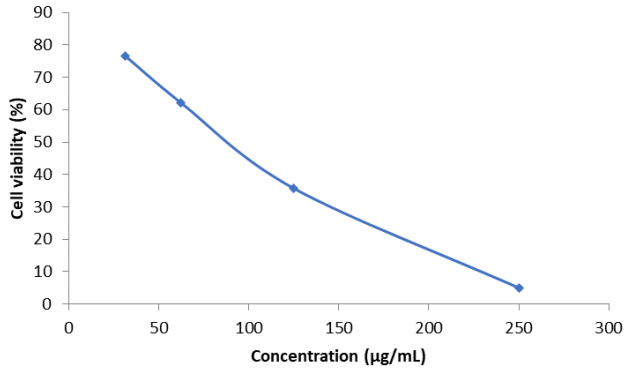
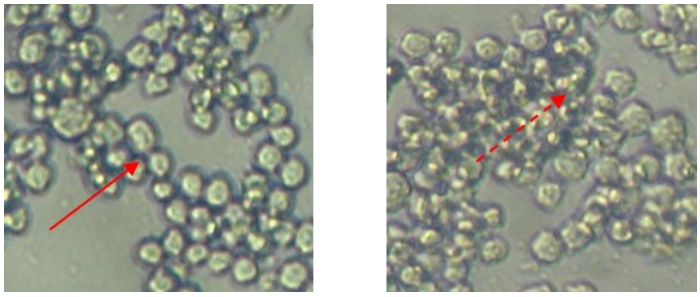


Figure 1. Effect of *P. angulata* L. extract on myeloma cells.

Furthermore, the inhibition activity of *P. angulata* L. extract against myeloma cells growth was also done using the MTT assay. The absorbance data using the MTT method illustrated the number of viable cells. Compounds that delay the doubling time of cell are thought to inhibit genes or proteins involved in the cell cycle. In this test, two concentrations of extract, below the IC_{50} value (15 and 30 µg/mL) were used. It was intended that not too many cells died in the observation for 72 hrs due to the cytotoxic effect of the extract. Observations were carried out at 24, 48, and 72 hrs. The results showed that *P. angulata* extract inhibited myeloma cell proliferation. The inhibitory effect of the extract on cell proliferation was directly proportional to its concentration (Table 1). The effect of cell proliferation inhibition from *P. angulata* extract was dose-dependent. These data indicated that treatment with extracts did not stop the cell cycle. This cell proliferation inhibition was related to cell cycle progression inhibition (Yu *et al.,* 2009). A previous study has also shown that the ethanol extract of *P. angulata* plants induces G_2/M arrest and apoptosis in the MDA-MB 231 human breast cancer and MCF-7 cell line (Hsieh *et al*., 2006).



A B

Figure 2. Morphology of myeloma cell before (A) and after (B) treatment at 125 µg/mL of P. angulata extract. Myeloma cells before being treated were round and clear while after the ethanol extract treatment (B), the cells were irregular in shape and dark in color.

Table 1. Inhibition effect of *P. angulata* L. ethanol extract in myeloma cell.

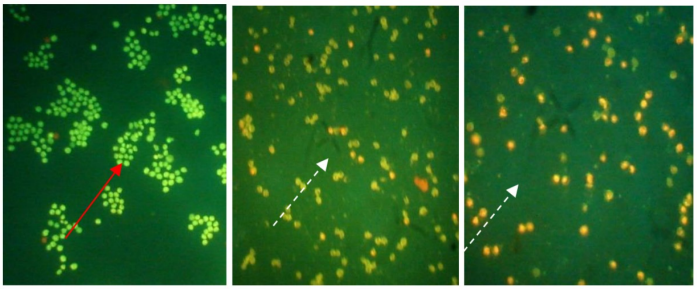
Time (hours)	Inhibition (%)		
	DMSO	Extract (15 µg/mL)	Extract (30 µg/mL)
24	5.53	17.83	25.41
48	5.78	24.1	45.04
72	7.36	26.16	53.90

Other studies have also found many compounds in plants capable of inducing cell cycle arrest and play an important role in prevention and cancer therapy. Soybeans contain genistein, daidzein, and isoflavonoids which play an important role in the prevention of breast cancer (Wang *et al*., 2002). Another flavonoid, such as quercetin, was able to inhibit cell cycle arrest at G2/M phase and induced apoptosis in human U937 cells (Lee *et al.,* 2006). Apigenin, the main flavonoids in celery exhibited antiproliferation effect on MCF-7 cells (Yin *et al*., 2001). Curcumin can induce the cell cycle arrest in the G_1 and G_2/M phases (Barti *et al*., 2003; Van Erk *et al*., 2003). PGV-0, a derivative compound of curcumin, has also been shown to inhibit T47D breast cancer cell proliferation (Meiyanto *et al*., 2006).

3.2 Apoptosis observation using double staining

Apoptosis is a cell suicide program to eliminate damaged or abnormal cells (Elmore, 2007). In this study, observations of cell DNA undergoing apoptosis were carried out by double staining using acridine orange-ethidium bromide (Kwan *et al*., 2015). Acridine orange will be absorbed by both viable and dead cells while ethidium bromide is only absorbed by cells that have lost membrane permeability. Acridine orange colors the living cells so that it appears green and ethidium bromide causes orange fluorescence in dead and apoptotic cells (Liu *et al*., 2015).

Cells undergoing apoptosis have a cell membrane with blebbing characteristics but do not lose their integrity, chromatin and nucleus membrane aggregation, cytoplasm shrinks, core condensation occurs, and cell fragmentation becomes apoptotic bodies (Wyllie *et al*., 2000; Liu *et al*., 2015). The results of double staining due to the administration of ethanol extract 50 µg/ml showed the presence of living cells and cells that might experience apoptosis as indicated by orange cells. Meanwhile, the treatment with 75 µg/mL ethanol extract showed a clearer apoptotic phenomenon (cells were orange and apoptotic bodies were formed) (Figure 3). These data indicate that the ethanol extract of *P. angulata* may stimulate apoptosis in myeloma cells.



A B C

Figure 3. Morphology of myeloma cell after double staining with ethidium bromide-acridine orange. Cell control, cells treated with extracts of 50 µg/mL, and cells treated with extracts of 75 µg/mL are shown in A, B, and C, respectively. In cell control, all visible green shows viable cells, whereas there were apoptotic cells (orange) in treated cells.

3.3 p53 and Bax expressions in myeloma cells

The p53 protein is a transcription factor of many genes which plays a role in apoptosis, cell cycle, and angiogenesis (Elmore, 2007). The DNA damage causes the p53 protein to increase the expression of proapoptotic proteins, such as Bax, NOXA, or Puma. Increasing the expression of proapoptosis proteins will stimulate the release of cytochrome C from mitochondria and stimulate apoptosis (Nakamura, 2004).

In this study, the expression of p53 and Bax proteins in myeloma cells after the treatment with *P. angulate* extract were observed using immunocytochemical staining using horseradish peroxidase enzyme-labeled antibodies. This enzyme reacted with chromogen DAB (diaminobenzidine) into a brown substrate. Cells with positive expressions had a brown/dark color due to the DAB and cells with negative expression had a purple or blue color due to counterstain hematoxylin. Positive protein expressions were compared with negative controls. In the negative control, the painting was done without primary antibodies so that the generated color was only purple or blue due to counterstain hematoxylin.

Results showed an increase in the expression of p53 and Bax proteins in myeloma cells (Table 2). It was suspected that apoptosis in Myeloma cells was caused by the increase in the expression of p53 and Bax proapoptotic proteins. Previous research reported that physapubenolide, a withanolide isolated from *P. angulata* induced apoptosis in human breast cancer MDA-MB-231 and MCF-7 cells by increasing the expression of Bax dan BCl_2 protein (Ma *et al.,* 2017). This is the first study reported the induction of p53 and Bax proapoptotic proteins by *P. angulata* extract in myeloma cells.

Table 2. Expression of p53 and Bax in myeloma cells in the control and treatment of 75 µg/mL *P. angulata* L. extract

Treatment	p53 Expression (%)	Bax Expression (%)
Control	18	58
75 µg/ml of extract	50	92

4. Conclusion

P. angulata has the potential to inhibit the proliferation in myeloma cell with the IC_{50} value of 70.92 µg/mL. Apoptosis in myeloma cell was also induced in *P. angulata*. The results suggested that ethanolic extract of *P. angulata* may be possible to be developed as blood cancer drug.

Conflict of Interest

The authors declare no conflict of interest.

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