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ANTICANCER ACTIVITY OF BANGLE HANTU (*Zingiber ottensii* Val.) RHIZOMES ON BREAST CANCER CELL LINES MCF-7

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ABSTRACT

Bangle Hantu (*Zingiber ottensii* Val.) is one of underutilized Zingiberaceae plants which are abundantly grow in Indonesia. It is not commonly used in Indonesian traditional medicine nor as spice in Indonesian culinary system. To increase its utilization, in this study we investigated anticancer activity of the methanolic extract of the rhizomes on human breast cancer cell line MCF-7. The antiproliferative effect was measured by comparing the rate of proliferation (doubling time) of cells treated with extract to untreated or control cells using *in vitro* tetrazolium salt (MTT) assay, and the ability to induce apoptosis was observed using acridine orange-ethidium bromide staining. The results showed that the methanolic extract significantly inhibit the proliferation of MCF-7, showed by no proliferation occurred in cells treated with 20 µg/mL extract (the lowest concentration used in the experiment) while only 25% cells left alive after 24 hours incubation with 80 µg/mL extract (the highest concentration used in the experiment). The extract also showed ability to induced apoptosis of MCF-7 cells. From the results we concluded that the methanolic extract of the rhizome of *Zingiber ottensii* Val. have anticancer activity and could be developed further as source of novel natural anticancer agent.

Keywords

Anticancer, *Zingiber ottensii*, MCF-7, antiproliferative, apoptosis.

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INTRODUCTION

Plants have been a source of medicine for thousands of years and phytochemicals continue to play an essential role in medicine. Zingiberaceae is a large family comprises more than 1000 species, and many of its member were famous as spices and medicinal plants as well. These plants thrives in tropical region such as Indonesia. One of the plants which abundantly grows wild in Indonesia is Bangle Hantu (*Zingiber ottensii* Val.). Despite of its abundance and wide distribution, Bangle Hantu is still underutilized, it is not commonly used in Indonesian traditional medicine nor as spice in Indonesian culinary system, maybe due to its unpleasant taste and odour. Very rare information was found about the use of this plant as traditional medicine, some of which mention about the use of Bangle Hantu as pain reliever, and sometimes use to cure fever and cough especially for children (Sinaga et al, 2000).

Rhizomes of Bangle Hantu contain essential oils, flavonoids, steroids, tannins, and other bioactive compounds. Flavonoids are well known for its strong antioxidant activity and some of them have been shown to have significant anticancer activity (Chahar et al, 2011; Naphong et al, 2013; Vijayalakshmi et al, 2013). Essential oil in Bangle Hantu rhizomes contains mainly zerumbon (37 to 40.1%), terpinen-4-ol (11.2 to 16.8%), α -humulen (5.6 to 10.9 %) and sabinen (6.5-7.2%) (Malek et al, 2005; Thubthimthed et al, 2005). Zerumbon isolated from *Zingiber zerumbet* L. had been shown to inhibit the growth of Human HeLa Cervical Cancer Cells, pancreatic cancer cells Panc-1, and HepG2, and also induced their apoptosis (Sakinah et al, 2007; Abdul et al, 2009; Zhang et al, 2012). In addition, the rhizomes of Bangle Hantu also contain a cysteine protease, called zingipain, which has

antiproliferative activities against fungi and human malignant cell lines (Karnchanat et al, 2011). Sinaga et al (2011) reported the strong cytotoxicity of the methanolic extract of Bangle Hantu rhizomes against human breast cancer cell lines MCF-7 with IC₅₀ value of 60 mg/ml, as strong as the methanolic extract of the rhizomes of *Zingiber zerumbet* L. (lempuyang gajah) and much stronger than methanolic extract of the rhizomes of *Nicolaia speciosa* L. (kecombrang). Several research groups have shown that *Zingiber zerumbet* rhizomes extract has strong anticancer activity (Rashied and Pihie, 2005; Ruslay et al, 2007; Yob et al, 2011). Therefore it is very intriguing to know whether Bangle Hantu, the close relative of *Zingiber zerumbet*, also has strong anticancer activity, especially in inhibiting proliferation and induced the apoptosis of cancer cells, so it can be used as source or raw material for a novel natural anticancer drug.

Materials and Methods

Plant material, cell line and chemicals

Fresh rhizomes of *Zingiber ottensii* Val. were obtained from BALITTRO (Balai Penelitian Rempah dan Tumbuhan Obat) Cimanggu Bogor, West Java. MCF-7 cell line were obtained from CCRC (Cancer Chemoprevention Research Center) Gadjah Mada University, Yogyakarta. BSA (Bovine Serum Albumin), PBS (Phosphate Buffer Saline), MTT Solution, Ethidium Bromide and Acridine Orange were purchased from Sigma-Aldrich Corp, St. Louis, MO, USA. DMEM (Dulbecco's Modified Eagle Media), FBS (Foetal Bovine Serum), 1% (v/v) Penicilline-Streptomycine Solution, and Tripsin-EDTA 0,25% were purchased from Gibco, Invitrogen Corporation, Grand Island, NY, 14072, USA.

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Preparation of plant extract

Slices of fresh rhizomes of Bangle Hantu (ca 10 kg) were sun dried for 2 days and autoclaved in an electric oven at 40°C for 5 days. The dried rhizomes slices were grinded and sieved with a 18 mesh sieve. The dried powdered (1000 g) of the rhizomes were extracted in room temperature with methanol (1.5 L) for 24 hours and then filtered. The process of extraction was repeated three times, and then the filtrat collected was concentrated by rotary vacuum evaporator.

Antiproliferative activity assay

Antiproliferative activity was measured by comparing the rate of proliferation (doubling time) of extract-treated cells with untreated or control cells by means of growth inhibition assay using *in vitro* tetrazolium salt (MTT) as described recently (Hamedeyazdan et al, 2012). Stock extract solution were prepared by dissolving 5 mg of extract with 100 mL DMSO. Stock solution was then diluted with DMEM medium to obtain a series of test extract solution.

MCF-7 cells were maintained in a humidified incubator with 5% CO₂ for 24 hours at 37°C. When the cells were 80-90% confluent, they were harvested by treatment with a solution containing 0.25% trypsin, thoroughly washed and resuspended in supplemented growth medium and seeded at a density of $\sim 5 \times 10^3$ per well. After 24 hours, to each well was added 100 μ L test solution with varying concentrations range from 20-80 μ g/mL, and then incubated for 6, 12, 24, 48 and 72 hours. 1.25% DMSO solution was used as control. At the end of incubation, the culture medium was removed by carefully aspirated, and cells were washed with 100 μ L PBS (Phosphate Buffer Saline). To each well then added 100 μ L of culture medium and 10 μ L of 5 mg/mL MTT solution, and the cells were reincubated for

next 6 hours. Viable cells will react with MTT to form purple-blue formazan. The reaction was stopped by MTT stopper reagent (Sodium dodecyl sulfate), shaken in shaker plate for 10 minutes, then incubated overnight in the dark at room temperature. Finally the absorbance of formazan in each well was measured with an ELISA reader at 595 nm. The resulting growth data represents the net outcome of cell proliferation. The cell viability (%) was obtained by comparing the absorbance between the samples and a negative control.

Apoptosis induction assay

The induction of apoptosis was observed by acridine orange/ethidium bromide (AO/EB) staining to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis as described by others (Ćurčić *et al*, 2012; Lakshmi *et al*, 2011) with a slight modification. MCF-7 cells grown on coverslips inserted in 24-well microplate to obtain a density of 5×10^4 cells/well and incubated until 50-60% confluent. After the cells were incubated with the extract for 48 hours with 1.25% DMSO as control solution, culture medium was removed by carefully aspirated, and cells were washed with PBS (Phosphate Buffer Saline). Cells were then stained with acridine orange-ethidium bromide solution and allowed to stand for 5 minutes, and then immediately observe under fluorescence microscope (Zeiss MC 80). Viable cells showed normal bright green nuclei, early apoptotic cells showed condensed green nuclei, dead cells (late apoptotic cells) showed condensed red-orange fluorescens nuclei, while necrotic cells showed normal red-orange fluorescens (McGahon *et al*, 1995). Number of apoptotic cells were observed and counted to quantify apoptosis.

Results

1 Antiproliferative activity of *Zingiber ottensii* Val. rhizomes

Methanolic extract of *Zingiber ottensii* Val. rhizomes possessed strong antiproliferative activity as shown in Figure 1. Observations were carried out for 72 hours at 0, 6, 12, 24, 48 and 72 hour, and the concentration of the extract used was around its cytotoxic-IC₅₀ value (60 µg/mL) revealed from previous experiment (Sinaga *et al.*, 2011). All concentration of extract used in this experiment (20-80 µg/mL) significantly inhibited the proliferation of MCF-7 cells.

The lowest concentration of Bangle Hantu rhizome's extract used in the experiment (20 µg/mL) had significantly inhibited the proliferation of MCF-7 cells from the beginning of incubation until the end of experiment, while the highest concentration (80 µg/mL) of extract showed strong cytotoxicity with only 25% cells left alive after 24 hours incubation, and almost all the cells dead after 72 hours incubation (Figure 1). At the same time, solvent-treated cells (negative control) showed an increase in cell's number (data not shown), means the proliferation occurred normally.

Apoptosis induction activity of *Zingiber ottensii* Val. rhizomes

Double staining with acridine orange and ethidium bromide revealed that methanolic extract of *Zingiber ottensii* Val. rhizomes had the ability to induce apoptosis on MCF-7 cell lines, showed by orange-red fluorescent cells (indicating by red arrow), that are characteristic of apoptotic cells (Fig. 2B).

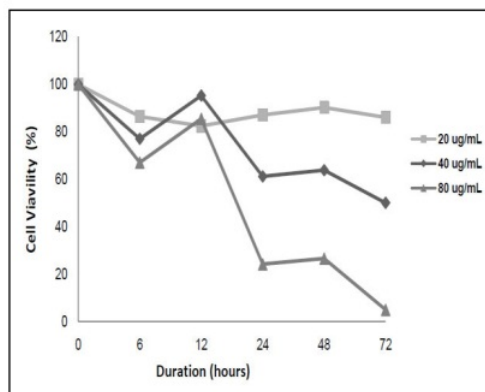


Figure 1. Inhibition of MCF-7 cells proliferation by methanolic extract of Bangle Hantu rhizomes. All concentration used in this experiment were effective in inhibiting the proliferation of the cells.

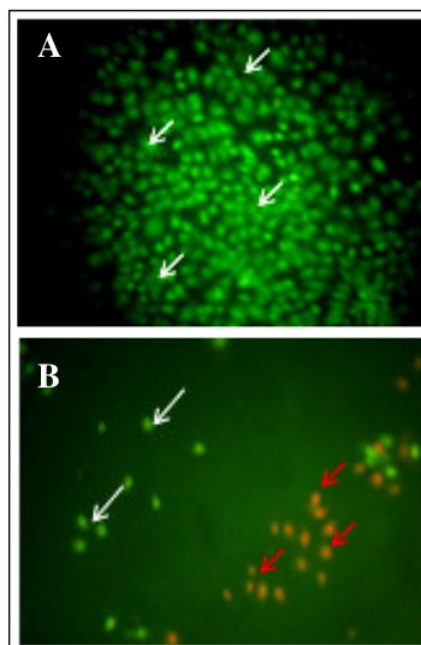


Figure 2. Treatment with methanolic extract of Bangle Hantu rhizomes induced apoptosis of MCF-7 cells. The untreated cells appeared in uniformly bright green colour indicating the normal living cells (A), while some of the treated cells had undergone apoptosis showed by orange-red fluorescent cells indicated by the red arrows (B).

1 Some cells underwent nuclei condensation shown by the yellow color in the nucleus, indicating early events of apoptosis, while the untreated cells showed uniformly bright green colour indicating viable cells (Figure 2A). The data obtained showed that MCF-7 cells were undergoing apoptosis after incubation for 24 hours, and increasing the concentration would increase the apoptosis.

Discussion

Rapid and uncontrolled proliferation is main characteristic and the primary key in the progression of tumor or cancer. Therefore, the ability of a substance to inhibit or suppress the proliferation of cancer cells is an important feature or property of a potential cancer drug. In the search for new cancer drugs derived from nature, in the present study we investigated the antiproliferative activity of methanolic extract of Bangle Hantu rhizomes on human breast cancer cell line, MCF-7, and also its ability to induce apoptosis of the cells.

Figure 1 clearly showed that the cells treated with methanolic extract of Bangle Hantu rhizomes showed a decrease in viable-cell's number from the beginning of the experiment until 72 hours incubation. At the lowest concentration used in the experiment, i.e. 20 µg/mL, the cells failed to doubled its number. It means that during the experiment some of the cells died, while proliferation did not occurred or significantly reduced.

The higher the concentration, the stronger the cytotoxicity of the extract. 24 hours incubation in 40 µg/mL extract caused cells dead with only 60% of the original number of viable cells left, while 24 hours incubation in 80 µg/mL extract only left 25% of viable cells. At the end of the experiment, i.e. at 72 hours incubation with 80 µg/mL extract, almost no viable cells could be observed (Fig. 1). These data corroborate the results of cytotoxicity

assay that had previously been conducted by Sinaga et al (2011), and revealed the antiproliferative activity of methanolic extract of Bangle Hantu rhizomes. However, in this experiment, the doubling time of MCF-7 cells treated with Bangle Hantu extract could not be calculated due to the very strong toxicity of the extract. Therefore the determination of doubling time should be done with other methods, such as by AgNOR staining or by using a lower concentration of extract, i.e. below 20 µg/mL.

Apoptosis is a genetically directed process of cell self-destruction, also called *programmed cell death*. Malignant or cancer cells generally lack of apoptosis. Induction of apoptosis is a useful marker for screening compounds for subsequent development as possible anticancer agents. Therefore it is very interesting to know whether methanolic extract of Bangle Hantu rhizome also posses ability to induce apoptosis.

Further experiment by double staining the MCF-7 cells with acridine orange and ethidium bromide demonstrated that the dramatically growth inhibition of methanolic extract of Bangle Hantu rhizomes on MCF-7 cells was caused, at least partly, by the apoptosis induced by the extract. Figure 2B showed some of the treated cells had undergone apoptosis shown by orange-red fluorescent. Acridine orange is a vital dye that stain both live and dead cells, whereas ethidium bromide stain only those cells that have lost their membrane integrity or apoptotic cells. Viable cells will appear uniformly green, while apoptotic cells underwent membran-blebbing and would incorporate ethidium bromide and therefore stain orange (Kasibhatla *et al*, 2006).

Results of the experiments revealed the potentiality of substances contained in the methanolic extract of Bangle Hantu rhizomes to strongly inhibit the proliferation of human breast cancer cell lines, MCF-7, and also

1 significantly induced the apoptosis of those cells. This means that methanolic extract of Bangle Hantu rhizomes have potential anticancer activity.

However, the extract used in this experiment was crude methanolic extract. It is necessary to isolate the active substances from the extract to determine which substance or substances, among abundant bioactive substances contained in the extract, are the most potent to be developed further as novel anticancer agents.

Conclusion

The study concludes that methanolic extract of the rhizomes of *Zingiber ottensii* Val. have anticancer activity due to its strong antiproliferative activity and ability to induced apoptosis in MCF-7 cell lines. Therefore it should be studied and developed further as source of natural anticancer agent.

Due to the lack of data on anticancer activity of *Zingiber ottensii* Val. rhizomes, it is necessary to conduct further research to examine the anticancer effect of the extract and its fractions on various cancer cell lines and to conduct *in vivo* study as well. It is also important to reveal the mechanism of action of anticancer compounds contained in the plant's part.

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