

ANTICANCER ACTIVITY OF ZINGIBER OFFICINALE AND PIPER RETROFRACTUM EXTRACT COMBINATION ON HELA CELL LINE

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Abstract

The use of chemotherapy for cancer cause side effects since its affect to synthesis of nucleic acids and proteins both cancer cells and normal cells. The combination of plants used to enhance the anticancer activity and to minimize side effects caused by anticancer drug. Red ginger rhizome (*Zingiber officinale* cv. Rubrum) and java pepper fruit (*Piper retrofractum*) are empirically used as an anticancer.

This study was conducted to investigate the cytotoxic and apoptotic activity of *Zingiber officinale* and *Piper retrofractum* extract and its effect to p53 and caspase 9 expression on HeLa cervical carcinoma cell. Cytotoxic assay was performed using MTT assay, apoptotic assay was performed by immunofluorescence method, using fluorochromes ethidium bromide and acridine orange. Expression of p53 and caspase 9 was examined by immunohistochemistry method. Cisplatin was used as positive control.

The result showed that extract combination has cytotoxic activity on HeLa cell line, with IC₅₀ = 33.81 µg/ml, through apoptotic mechanism, increase expression of p53 tumor suppressor gene and caspase 9.

Keywords: *Zingiber officinale*, *Piper retrofractum*, cytotoxic, HeLa Cell Line

INTRODUCTION

World Health Organization (WHO) provides an illustration that 12% of all deaths in the world caused by cancer. Cervical carcinoma is the leading cause of cancer-related death in women (Suwiyoga, 2007). Based on data from the Ministry of Health of the Republic of Indonesia in 2005, the incidence of cervical carcinoma was ranked

at second position after breast cancer (Anonymous, 2007). Cancer chemotherapy are include cytostatic drugs, hormones, antihormon, and biological compounds. These drugs cause side effects on the synthesis of nucleic acids and proteins, so the cancer cells and normal cells will be damaged (Stetler and Kleiner, 2001).

Nowadays, many researchs conducted to discover new drugs for cancer therapy. Indonesia is rich in natural resources, particularly plant

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materials, which are used empirically for cancer, such as red ginger (*Zingiber officinale* cv. Rubrum) and java pepper fruit (*Piper retrofractum*). Red ginger rhizome have been studied for anti-cancer activity and anti-inflammatory on liver cancer (Habib et al., 2008), inhibit the activity of cell growth and modulates secretion angiogenic factor in ovarian cancer cells (Rhode et al., 2007). Kim et al. (2008) stated that the red ginger rhizome have active content as a cytotoxic agent, namely oleoresin consisting of gingerol, paradol, shogaol, zingerone, resin and volatile oil which is a group of terpenoids (Ravindran et al., 2005).

Java pepper has been investigated for antioxidant activity (Jagdale et al., 2009; Wakade et al., 2008), analgesic activity to reduce pain in cancer (Febrina and Subarnas, 2006), and cytotoxic effects on cells Myeloma with IC₅₀ of 55,48 µg/mL (Setyorini, 2007). The anticancer active compound in java pepper fruit are alkaloids (Selvendiran et al., 2003; Pradeep and Kuttan, 2004).

The research of the effect of *Piper chaba* Linn, *Piper sarmentosum* Roxb, *Piper interruptum* Opiz., *Plumbago indica* Linn. and *Zingiber officinale*) against breast adenocarcinoma cells (MCF-7) obtained IC₅₀ respectively 35.17; 31.15 and 33.20 µg/mL.

Apoptosis (programmed cell death) is one of mechanism caused cytotoxic activity. The p53 gene appears to trigger apoptosis through regulating uncontrolled cellular proliferation in the setting of aberrant growth signals. Mutations in

the p53 gene result in loss of the ability of the gene product to bind to DNA, thereby removing its suppressive effect (Rugo, 2006). When cellular stress (e.g. DNA damage) occurs, proapoptotic proteins in the cytosol will be activated. As a result, cytochrome c localized in mitochondria will be released to the cytosol, activated caspase-9 (Fan et al., 2005).

The purpose of this study was to investigate the cytotoxic and apoptosis activity of the combination ginger rhizome and piper java fruit ethanolic extract on HeLa cells and its effect on p53 and caspase 9.

MATERIAL AND METHODS

Plant materials, chemicals, cell line culture

Z. officinale rhizome and *P. retrofractum* were purchased on the local market and botanical identification in laboratory at biology faculty, Jenderal Soedirman University and stored as a voucher specimen in the same faculty. Cisplatin were obtained from parasite laboratory, Faculty of Medicine, Gadjah Mada University. HeLa cervical cancer cells were obtained from parasite laboratory, Faculty of Medicine, Gadjah Mada University. HeLa cells were routinely cultured in RPMI 1640 medium (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA) at 37°C in a 5% CO₂ atmosphere, 3% penicillin streptomycin and 1% fungison. Subcultures were obtained after treatment with 0.05% trypsin (Gibco, Auckland) in phosphate buffer saline.

Preparation of extract combination

Red ginger and java pepper was, cut into pieces, dried and crushed into powder. 400 grams of red ginger rhizome powder, 400 grams of java pepper 1:1 (each 200 gr) were extracted by maceration using 96% ethanol for 3 x 24 hours. The extract is filtered and then evaporated.

Cell viability assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

For cell viability assay, 1.5×10^4 cells/well were plated in 100 μ l of RPMI 1640 media. Cells were incubated overnight at 37°C in humidified atmosphere of 5% CO₂ for cells attachment. After 24 h cell incubation, extract was added at various concentrations ranging from 500; 250; 125; 62.5; 31.25; 15.63; and 7.81 μ g/mL. After 24 h of incubation, 10 μ L MTT reagen of 5 mg/mL in PBS was added to the plate. The resulting MTT-products were determined by measuring the absorbance at 595 nm with ELISA reader. Each point represents the mean of triplicate experiments. Absorbance data then calculated to the standard curve equation of HeLa cells in order to get the number of living cells in the control cells and the number of living cells in the test cell.

Determination of Apoptosis.

HeLa cells were grown on glass coverslips in tissue culture dishes (Falcon) and were allowed to attach for 24 h prior to the addition of extract. After the cells were incubated with extract for 24 hours, the coverslips were washed once in phosphate-buffered saline and fixed in object glass. Treated cells were stained with acridine orange and ethidium bromide 5 μ L and visualized

by fluorescence microscopy. Viable (normal, green nuclei), early apoptotic (condensed, green nuclei) and late apoptotic (condensed, red nuclei) cells were counted.

Expression of p53 and caspase-9.

HeLa cervical cancer cells were cultured in a 24-well plates (Nalge Nunc International, Denmark) at a density of 1.5×10^5 cells per well, incubated 24 h.

The cells were then treated with 1x IC₅₀, 0.5x IC₅₀, 0.25x IC₅₀ combination extract and cisplatin. After 24 h, cells were plated in poly-L-Lisin slide. Cells were fixed with methanol (pro analysis) for 5 minutes, permeabilized for 5 min in PBS containing 0.2% Triton X-100, blocked in 2% BSA for 1h, and stained with the monoclonal antibody p53 or caspase-9 (1:400) for 1 h, further washed with PBS 3x 5 min, stained with bionylated secondary antibody for 1 h. Cell was incubation in HRP-streptavidin for 10 minutes, added DAB for 5 min, washed with aquadest, then counterstained with Harry's hematoxylin for 20 sec, mounted on glass slides. The cells were examined using a confocal microscope.

STATISTICAL ANALYSIS

Data were analyzed as Annova determine statistically significant differences at the P d" 0.05. All analyses were conducted using SPSS for Window.

RESULTS AND DISCUSSION

HeLa cells were treated with various doses of

ginger rhizome, java pepper fruit, extract combination and cisplatin, incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Cell viability was determined by MTT assay, absorbance was read at 595 nm. Inhibition rate (%) was defined as : ((live cell in the control – live cell in the test group)/live cell in the control) x 100. Standard curve : $y = 0.00001x + 0.207$ ($R^2 = 0.927$). Results are average of three independent experiments (mean ± SD).

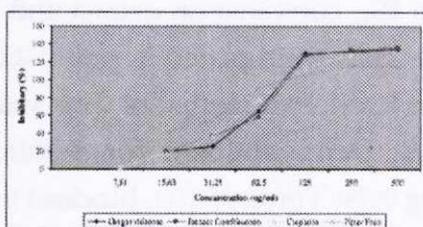


Figure 1. Inhibition rate on HeLa cell line with ginger rhizome, piper java fruit, extract combination and cisplatin

Figure 1. showed increased concentrations of test material causing an increase in the percentage inhibition of HeLa cell growth. The mean absorbance of the red ginger extract, java pepper fruit, and extract combination with a concentration 500 µg/mL are 0.027; 0.019; 0.050 respectively. While the concentration at 15.63 µg/mL are 0.625; 0.670, and 0.634. Concentrations of cisplatin at 125 and 3.905 µg/mL has a mean absorbance of 0.133 and 0.550 respectively. At a concentration of 62.5 µg/mL, red ginger rhizome extract, java pepper fruit, and extract combination inhibiting the growth of HeLa cells to 65.526%, 58.137% and 77.298%. At 15,63 µg/mL, Cisplatin inhibiting HeLa cells up to 91.237%.

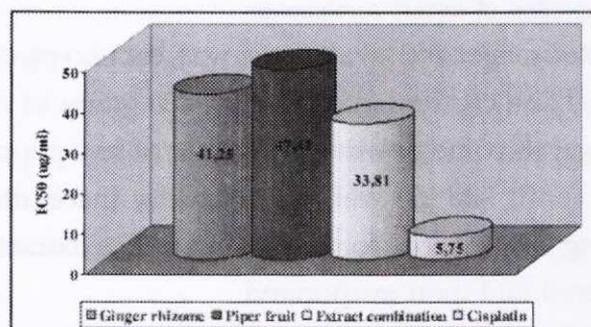


Figure 2. IC₅₀ of ginger rhizome, piper java fruit, extract combination and cisplatin on HeLa cancer cells. Concentrations inhibiting 50% of the cell were determined by probit analysis using SPSS software.

Figure 2. showed that the material has a ability to inhibit HeLa cell growth as shown by the IC₅₀ value. Extract combination (1:1) has IC₅₀ 33.81 µg/mL. This indicates that the combination has cytotoxic activity against HeLa cells. Meyer et al. (1982) declares an extracts said to have anticancer activity if the IC₅₀ value of less than 1000 µg/mL after 24 hours of contact time. IC₅₀ is concentration that can inhibit cell growth by 50% cell line. The smaller the IC₅₀ of a compound the more toxic compound it was (Doyle and Gaffiths, 2000).

Merging or combining several plants in cancer treatment performed to enhance the cytotoxic activity and could minimize side effects caused by the use of anticancer drugs (Beinfeld, 2005). Cytotoxic activity of extract combination against HeLa cells was higher with an IC₅₀ value of 33.807 compared with IC₅₀ values of each extract, i.e 41.249 and 47.409 µg/mL for red ginger rhizome and java pepper fruit extracts. This is due to red ginger and java pepper fruit has different mechanism against cancer. Red ginger rhizome could raise natural killer cell activity (NK) to lisis target cells, namely tumor cells and virus-

infected cells (Zakaria et al., 1999) and is able to inhibit the activity of NF κ B (Nuclear Factor kappa B) through the inhibition of cytokine pro inflammation, so the emergence of inhibit TNF- α which is the cause of the emergence of tumor (Habib et al., 2008; Hudson et al., 2000).

Piperine contained in java pepper fruit protect cells from cancer by binding proteins in the mitochondria to trigger apoptosis without harming normal cells through enhanced activity of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (Selvendiran et al., 2003). Additionally, piperine may inhibit NF κ B thereby preventing the formation of tumors through TNF- α , so angiogenesis does not occur (Pradeep and Kuttan, 2004). Given the different mechanism of action following the combination of plants can enhance the cytotoxic activity.

Red ginger rhizome extracts had IC₅₀ values up to 41.249 μ g/mL less than the piper java fruit extract of chili java 47.409 μ g/mL. Differences in mechanism of action against cancer cells affect the cytotoxic activity. Research carried out by Rhode et al. (2007) showed that the red ginger can inhibit cell growth and modulates secretion angiogenic factor in ovarian cancer cells. Therefore, a potential red ginger in the treatment and prevention of ovarian cancer. Another study carried out on liver cancer and metastases by inhibiting activation of CD8 + T cells (Habib et al., 2008; Suzuki et al., 1997). Red ginger were able to minimize the side effects of cancer drugs such as nausea and vomiting (Ernst and Pittler, 2000).

Java pepper fruit extract in this study have cytotoxic activity against HeLa cells with IC₅₀ of 47.409 μ g/mL. This showed that 96% ethanol extract of piper java fruit has cytotoxic activity against HeLa cells was better than the 70% ethanol extract of chilies java (Suhartatik, 2008). In addition, cytotoxic activity against HeLa cell of java pepper fruit extracts better than against myeloma cells. This is shown by IC₅₀ values of java pepper fruit against HeLa cells are smaller than the myeloma cells that is 46.246 μ g/mL and 55.48 μ g/mL. This difference in the chances of having the target compounds and causes of action of cancer cells in HeLa cells and myeloma cells. The study by Choi et al. (2009) showed that the piper fruit can reduce the risk of cisplatin resistance of cancer cells by induction of apoptosis via heme oxygenase-1 (HO-1). Cisplatin has the smallest IC₅₀ value of 5.745 μ g/mL. It showed better cytotoxic activity of cisplatin against HeLa cells.

Based on the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, cisplatin is the first-line monotherapy in the treatment of cervical cancer (Teng et al., 2004). IC₅₀ values for each group treated with three replication in the same time. The significance value greater than 0.05 means there is no significant difference among the four treatments.

Extract combination induced apoptosis and increase p53 tumor suppressor gene

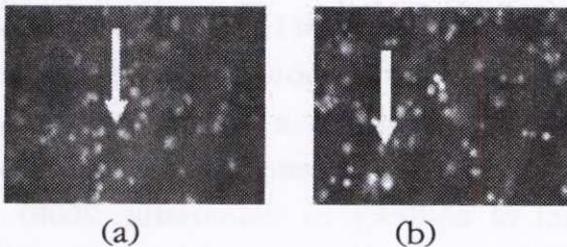


Figure 3. Cells were treated with acridine orange and ethidium bromide. Viable cell (orange arrow(a)) and apoptotic cell (white arrow(b)). HeLa cells treated with extract combination underwent the classical morphologic changes indicate of apoptosis

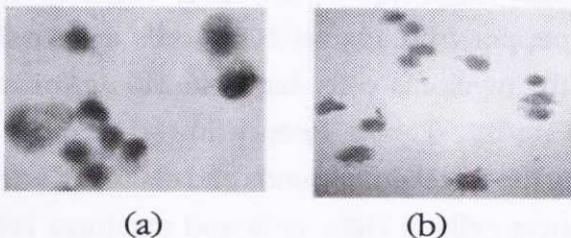


Figure 4. Expression of p53 on HeLa cancer cells. Untreated cell (a), extract combination (b), by immunohistochemistry method. Treated cells were plated on poly-L-lysine and stained with a p53-specific mAb

Ginger extract at increasing concentrations induced apoptosis dose dependently in colon cancer cells (Abdullah et al., 2010). [6]-gingerol associated with the modulation of p53 and involvement of mitochondrial signaling pathway in B[a]P-induced mouse skin tumorigenesis (Nigam et al., 2009). Java chilli fruit extract protects cells from cancer by binding proteins on cancer cell mitochondria to trigger apoptosis without damaging the surrounding cells through increased activity of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (Selvendiran et al., 2003). In addition, chillies java can inhibit NF κ B, inhibit tumor formation by TNF- α that resulted in no occurrence of angiogenesis (Pradeep and Kuttan, 2004)

Extract combination induced caspase 9 expression

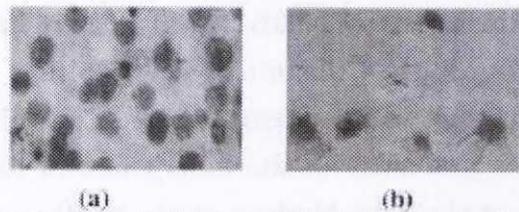


Figure 5. Expression of caspase 9 on HeLa cancer cells. Untreated cell (a), extract combination (b), by immunohistochemistry method. Treated cells were plated on poly-L-lysine and stained with a caspase 9-specific mAb

When cellular stress (e.g. DNA damage) occurs, proapoptotic proteins in the cytosol will be activated, which will in turn induce the opening of mitochondrion permeability transition pores (MPTPs). As a result, cytochrome c localized in mitochondria will be released to the cytosol. With the presence of cytosolic dATP (deoxyadenosine triphosphate) or ATP, apoptotic protease activation factor-1 (Apaf-1) oligomerizes. Together with cytosolic procaspase-9, dATP and cytochrome c, oligomerized Apaf-1 can result in the formation of a massive complex known as apoptosome. The N-terminal of Apaf-1 and the prodomain of procaspase-9 both have CARDs, with complementary shapes and opposite charges. They interact with each other by CARDs and form a complex in the proportion of 1:1. Activated caspase-9 can in turn activate procaspase-3 and procaspase-7. The activated caspase-3 will then activate procaspase-9 and form a positive feedback activation pathway (Fan et al., 2005). [6]-Gingerol upregulated the testosterone depleted levels of p53 in mouse prostate and upregulated its downstream regulator Bax and further activated Caspase-9 and Caspase-3 in both LNCaP cells and in mouse (Shukla, 2007).

Red ginger extract has anticancer effects and anti-inflammatory by preventing the activation of NF κ B (Nuclear Factor kappa B), preventing the translocation of NF κ B to the nucleus and prevent the dimer-dimer binding to DNA and block the bad effects of TNF- α (tumor necrosis factor alpha) causes inflammation (Habib et al., 2008). NF κ B is a stimulant in the form of signals that can activate normal cells, causing inflammation and carcinogenesis thus resulting in the appearance of TNF- α causes the appearance of tumors [Lin and Karim, 2003; Philip, 2004].

CONCLUSION

Ginger rhizome and java pepper fruit extract combination has cytotoxic activity on HeLa cell line, with IC₅₀ = 33.81 μ g/ml, caused cytotoxicity through apoptotic mechanism, increase p53 tumor suppressor gene and casapase 9 expression.

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