

***Zingiber officinale*, *Piper retrofractum* and Combination Induced Apoptosis and p53 Expression in Myeloma and WiDr Cell Lines**

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In previous studies, *Zingiber officinale*, *Piper retrofractum*, and the combination showed cytotoxic activity, induced apoptosis, and p53 expression of HeLa, T47D, and MCF-7 cell lines. This study was conducted to investigate the cytotoxic and apoptotic activity of *Zingiber officinale* (ZO), *Piper retrofractum* (PR), and the combination as well as their effect to p53 expression on Myeloma and WiDr cells. The powder of ZO, PR, and ZO + PR combination (1:1) were macerated with 96% ethanol for 3 x 24 hours. MTT cytotoxic assay was performed on Myeloma and WiDr cell lines. Apoptotic cells were stained with ethidium bromide and acridine orange. Immunohistochemical expression of p53 was examined on Myeloma and WiDr cell lines. Doxorubicin was used as positive control in all assays. Results showed that ZO, PR, and ZO + PR combination had cytotoxic activity on Myeloma cells with IC₅₀ of 28, 36, and 55 mg/ml respectively and WiDr cell lines with IC₅₀ of 74, 158, and 64 mg/ml respectively, induced apoptotic activity, and increased p53 expression on Myeloma and WiDr cells. These results suggest that ZO, PR, and their combination induced Myeloma and WiDr cells in apoptosis through p53 expression.

Key words: *Zingiber officinale*, *Piper retrofractum*, Myeloma Cell, WiDr cell

INTRODUCTION

Indonesia has a diversity of plant species with a potential for being medicinal plants. Two of these medicinal plants are red ginger (*Zingiber officinale*. var. *rubrum*) and javanese chili (*Piper retrofractum*).

Piperin (bioactive compound of *P. retrofractum*) and 6-gingerol (bioactive compound of *Z. officinale*) suppress inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis (Aggarwal & Shishodia 2006). Research, both *in vitro* and *in vivo*, has been done on *Z. officinale* and *P. retrofractum*. According to Habib *et al.* (2008), ZO can be used as an anti-cancer and anti-inflammatory by inhibiting Nuclear Factor kappa B (NFκB) and signaling the arrival of Tumor Necrosis Factor-α (TNF-α). Ginger extract inhibit cell proliferation of HCT 116 and HT 29 with IC₅₀ of 496 ± 34.2 μg/ml and 455 ± 18.6 μg/ml (Abdullah *et al.* 2010). While PR has benefits as an analgesic and antioxidant, there is study reporting that javanese chili has a toxic effect against myeloma cells with IC₅₀ of 55.48 μg/ml (Setyorini 2007). Wicaksono *et al.* (2009) stated that the methanol extract of *Piper crocatum* Ruiz and Pav leaves have the ability to inhibit T47D cells at IC₅₀ 44.25 μg/ml *in vitro*. The study of cancer treatment by combining several plants has been done in Thailand, with a herbal medicine called Pikutbenjakul. Sompokdeejaroen and Itharat (2009) reported that the cytotoxic activity of *Piper Chaba*, *Zingiber*

officinale, and Pikutbenjakul (*Piper Chaba*, *Piper sarmentosum*, *Piper interruptum*, *Plumbago indica*, and *Zingiber officinale*) against breast adenocarcinoma cells (MCF-7) obtained IC₅₀ *Piper Chaba*, *Zingiber officinale*, and Pikutbenjakul were 35.17; 31.15; and 33.20 μg/ml respectively.

The research on ZO, PR, and their combination for anticancer agent in myeloma and WiDr cancer cell has not been done. This research observed cytotoxic and apoptotic activity of ZO, PR, and its combination and their effect to p53 gene on Myeloma and WiDr cells.

MATERIALS AND METHODS

Plant Materials, Chemicals, Cell Line, and Culture.

Z. officinale rhizome and *P. Refractum* were obtained from the local market, authenticated, and stored in the Taxonomy Laboratory at the Biology Faculty of Jenderal Soedirman University. Doxorubicin was obtained from the Parasite Laboratory, Faculty of Medicine, Gadjah Mada University. Myeloma and WiDr cancer cells were obtained from Parasite Laboratory, Faculty of Medicine, Gadjah Mada University. Cultured myeloma was first taken from Merwin Plasma Tumor Cells-11 (MPC-11) isolated from murine Balb/c collection of J. Fahey in 1967. These myeloma cells resembled the parent tumor cells that produce IgG2b 5-6 g each cell/minute with a time division of approximately 17 hours (Terpos 2005). WiDr is a colon cancer cell in humans that was isolated from the colonic epithelial tissue of a 78-year-old woman (Chen *et al.* 1987). WiDr carsinoembryonic produce antigens and requires

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about 15 hours to be able to complete one cell cycle. One of the characteristics of WiDr cells is overexpression of cyclooxygenase-2 (COX-2) (Palozza *et al.* 2005). Myeloma and WiDr cells were routinely cultured in RPMI 1640 medium (Sigma) and supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA) at 37 °C in a 5% CO₂ atmosphere, 3% penicillin- streptomycin, and 1% fungizone. Subcultures were obtained after treatment with 0.05% trypsin (Gibco, Auckland) in phosphate buffer saline (PBS).

Preparation of Extract Combination ZO and PR were washed, cut into pieces, dried and crushed into powder. 400 g of ZO rhizome powder, 400 g of PR and combination of ZO:PR = 1:1 (one each 200 g) were extracted by maceration using 96% ethanol for 3 x 24 hours. The extract was 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 µl of culture media. Cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂ for cells attachment. Extracts were added at various concentrations ranging from 500 to 7.81 µg/ml. After 24 hours incubation, 10 µl of MTT solution (MTT reagent in 5 mg/ml PBS) was added to the plate. The resulting MTT-products were determined by measuring the absorbance at 595 nm with ELISA (Mosmann 1983).

Determination of Apoptosis. Myeloma and WiDr cells were grown on glass coverslips in tissue culture dishes (Falcon) and were allowed to attach for 24 hours prior to the addition of any drug. After the cells were incubated with a drug for 24 hours, the coverslips were washed once in PBS and fixed in object glass. Treated cells were stained with acridine orange and ethidium bromide 5 µl and visualized by fluorescence microscopy (Mosmann 1983).

Determination of p53. WiDr cancer cells were cultured in 24-well plates (Nalge Nunc International, Denmark) at a density of 1.5×10^5 cells per well and incubated for 24 hours. The cells were then treated with $1 \times IC_{50}$, $0.5 \times IC_{50}$, $0.25 \times IC_{50}$ concentrations of combination extract and doxorubicin. After 24 hours, cells were plated in *poly-L-Lysin* slides. Cells were fixed with methanol (pro analysis) for 5 minutes, permeabilized for 5 minutes in PBS containing 0.2% Triton X-100, blocked in 2% BSA for 1 hour, and stained with the monoclonal antibody p53 (1:400) for 1 hour. It was then washed with PBS for 3 x 5 minutes and stained with *bionylated secondary antibody* for 1 hour. Next, it was incubated in HRP-streptavidin for 10 minutes, added DAB for 5 minutes, washed with aquadest, *Counterstained* with *Harry's hematoxylin* for 20 seconds, and mounted on glass slides.

RESULTS

ZO, PR, and the combination extract had cytotoxic activity on myeloma cells with IC₅₀ values of 28, 36, and 55 µg/ml respectively. In this study, doxorubicin inhibited cells with IC₅₀ values of 2 µg/ml (Figure 1). ZO, PR, and the combination extract also had cytotoxic activity against

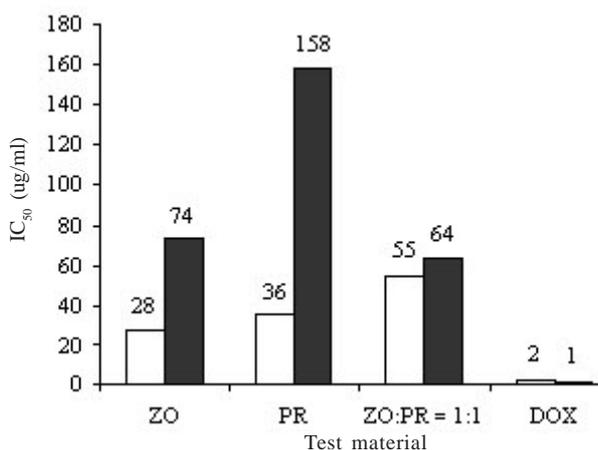


Figure 1. IC₅₀ of *Z. Officinale* (ZO), *P. retrofractum* (PR), extract combination and doxorubicin (DOX) on Myeloma and WiDr cancer cells. Cells were treated with various doses of ZO, PR, extract combination and DOX, incubated for 24 hours at 37 °C in humidified 5% CO₂ atmosphere. Cell viability was determined by MTT assay, absorbance was read at 595 nm. Concentrations inhibiting 50% of the cell were determined by probit analysis using SPSS software. □: Myeloma, ■: WiDr

the cancer cell WiDr. This was indicated by the IC₅₀ value of 74 and 158 µg/ml respectively. The combination extracts have cytotoxic activity with IC₅₀ value of 64 µg/ml and doxorubicin 1 µg/ml (Figure 1). The apoptosis assay results being viewed under a microscope are shown in Figure 2.

Results showed there was no apoptosis activity in myeloma and WiDr cell control (Figure 2a,e) shown as green cells. Myeloma cells that were treated with extract (Figure 2b,c,d), resulted in an orange color. This showed that the treated cells were induced into apoptosis. WiDr cells that were treated with extract (Figure 2f,g,h), resulted in an orange colour. This indicated that the cells were induced into apoptosis.

P53 immunocytochemistry of treated cells can be determined qualitatively from the figure in the microscope (Figure 3). Cells that express p53 protein are marked with brown cell nuclei and cells that do not express the p53 protein are marked with a purple nucleus. P53 protein expression results are viewed under a microscope.

DISCUSSION

Figure 1 showed that the extracts had cytotoxic activity on Myeloma and WiDr cells, as shown by the IC₅₀ value. This indicated that the extract had cytotoxic activity against Myeloma and WiDr cells. Meyer *et al.* (1982) declared that the extracts have anticancer activity if the IC₅₀ value were less than 1000 µg/ml after 24 hours of contact time. The smaller the IC₅₀ of a compound, the more toxic the compound = was (Doyle & Griffiths 2000).

Merging or combining several plants in cancer treatment was performed to enhance the cytotoxic activity and minimize side effects caused by the use of anticancer drugs (Beinfeld & Kornglod 2005; Sapakdeejaroen & Itharat 2009). Cytotoxic activity of the combination extract

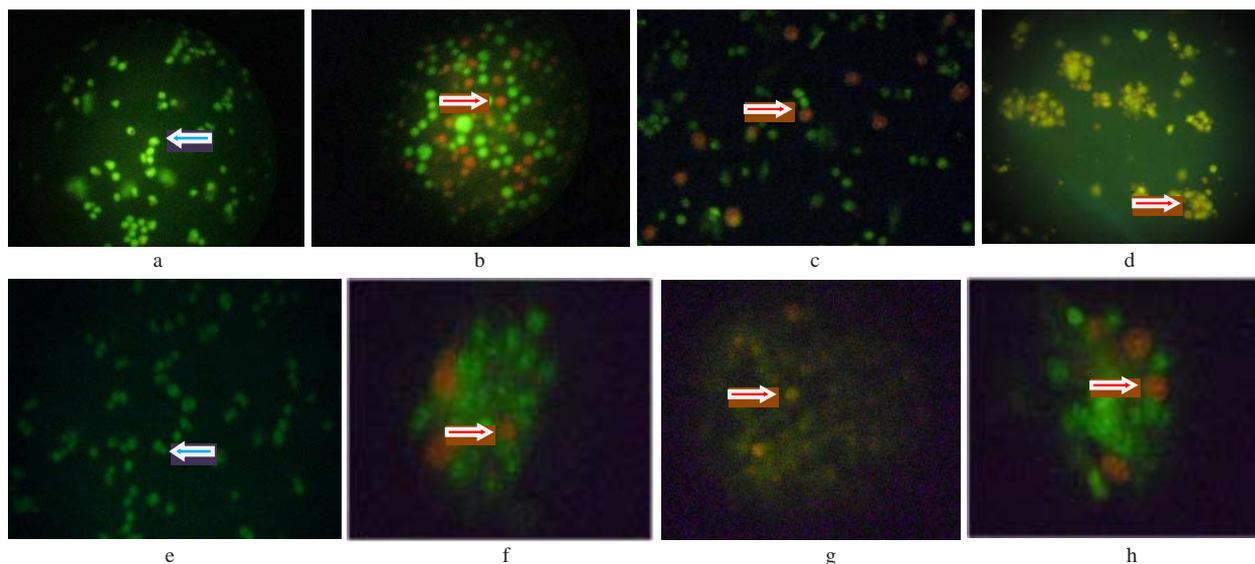


Figure 2. Apoptosis activity of *Z. Officinale*(ZO), *P. retrofractum* (PR) and extract combination. Cells were treated with acridine orange and ethidium bromide. All cells in the control were in a green color. In the treatment with ZO, PR and its combination extract, most cells were in a green color and some were in an orange color. Green color showed viable cells (blue arrow) (a,e) and apoptotic cells (red arrow) were shown in orange. Myeloma (b,c,d) and WiDr cells (f,g,h) treated with ZO, PR, and its combination extract in IC_{50} value respectively, underwent a color change from green to orange indicating apoptosis.

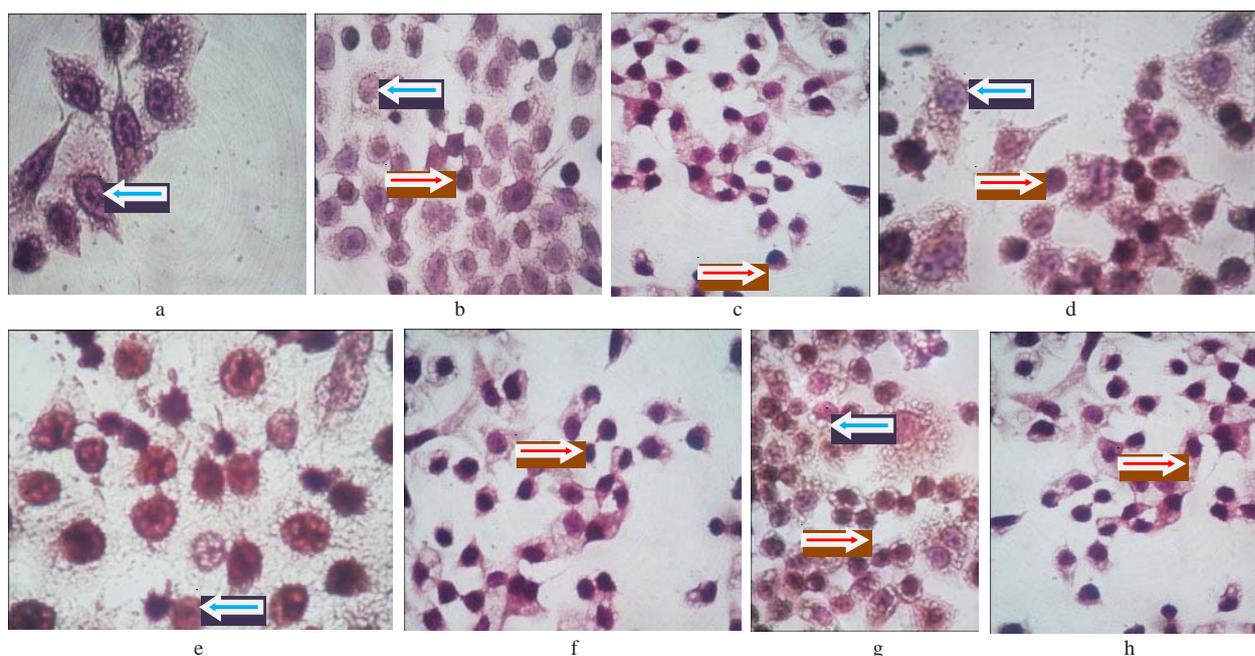


Figure 3. Expression of p53 on Myeloma and WiDr cancer cells. Untreated cell (a,e), treatment with ZO, PR, and its combination on Myeloma cells (b,c,d) and on WiDr cells (f,g,h) by immunohistochemistry method. The expression of p53 showed dark colour (purple to black, red arrow) in the nuclei. The blue arrow showed cells without p53 expression. Cells were plated on poly-L-lysine and stained with a p53-specific mAb.

against WiDr cells was higher with an IC_{50} value of $64 \mu\text{g/ml}$ compared with IC_{50} values of each individual extract, specifically 74 and $158 \mu\text{g/ml}$ for ZO and PR extracts respectively. In Myeloma cells, the combination extract has an IC_{50} value of $55 \mu\text{g/ml}$. This was caused by the different mechanisms of ZO and PR against cancer. ZO could raise natural killer cell activity (NK) to lyse target cells, namely tumor cells and virus-infected cells and was able to inhibit the activity of NF κ B through the inhibition of cytokine pro-inflammation (Hudson *et al.* 2000; Habib

et al. 2008). Research carried out by Rhode *et al.* (2007) showed that ZO extract inhibited cell growth and modulated secretion of angiogenic factor in ovarian cancer cells. Another study carried out on liver cancer showed that ZO inhibited the activation of CD8⁺ T cells (Suzuki *et al.* 1997; Habib *et al.* 2008). NF κ B is a stimulant in the form of signals that can activate normal cells, causing inflammation and carcinogenesis (Lin & Karin 2003; Philip *et al.* 2004). The existence of this NF κ B may lead to new agents that are TNF- α . TNF- α is the cause of the

emergence of tumors in various experimental models of carcinogenesis (Philip *et al.* 2004). Thus, inhibiting NFκB signal is a strategy for treating cancer (Kim *et al.* 2008).

Piperine contained in PR 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 NFκB thereby preventing the formation of tumors through TNF-α, so angiogenesis does not occur (Pradeep & Kuttan 2004). Given the different mechanism of action, the combination of plants can enhance cytotoxic activity.

Figures 2 and 3 showed that ZO, PR, and the combination extract induced apoptosis and p53 expression. Apoptosis is characterized by distinct morphological changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Ward *et al.* 2008). The process of apoptosis in Multiple Myeloma has problems due to the occurrence of metastases in the bone marrow which is the result of an imbalance in proliferation and apoptosis processes (Scudla 2005). This study was similar with study done by Abdullah *et al.* In 2010 which showed that ginger extract at increasing concentrations induced apoptosis, dose dependently, in colon cancer cells. [6]-gingerol was associated with the modulation of p53 and the involvement of mitochondrial signaling pathway in B[a]P-induced mouse skin tumorigenesis (Nigam *et al.* 2009). Ginger can induce apoptosis via mitochondria involving caspase-8, BID cleavage, cytochrome c release and caspase-3 activation, such as curcumin. ZO and PR can affect the activity of NFκB. A few NFκB regulated genes, including Bcl-2, Bcl-XL, cIAP, TRAF1, and TRAF2, have been reported to function primarily by blocking the apoptotic pathways (Aggarwal & Shishodia 2006).

Research carried out by Rhode *et al.* (2007) showed that red ginger can inhibit cell growth and modulate the secretion angiogenic factor in ovarian cancer cells. In addition, piperin can inhibit NFκB, which inhibits tumor formation by TNF-α resulting in no occurrence of angiogenesis (Pradeep & Kuttan 2004).

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