

Efek fraksi protein daun *Carica papaya* L. pada ekspresi protein p53 dan Bcl-2 pada kultur sel kanker payudara

Effect of protein fraction of *Carica papaya* L. leaves on the expressions of p53 and Bcl-2 in breast cancer cells line

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Abstrak

Daun *Carica papaya* L diketahui mengandung protein RIP dan mempunyai aktivitas sitotoksik secara *in vitro* terhadap kanker sel. Penelitian ini bertujuan untuk mengetahui efek fraksi protein tersebut pada ekspresi protein p53 dan Bcl-2 yang merupakan protein regulator apoptosis.

Fraksi protein daun *Carica papaya* L. Diperoleh dengan jalan melakukan pengendapan menggunakan amonium sulfat. Fraksi pengendapan yang diperoleh, diuji aktivitasnya dalam memotong DNA superkoil untai ganda untuk memastikan adanya RIP. Selanjutnya fraksi protein diuji aktivitasnya pada sel kanker payudara dengan menentukan efek sitotoksitas secara *in vitro* pada, induksi apoptosis dengan pengecatan DNA dan analisis ekspresi protein regulator apoptosis, p53, Bcl2, menggunakan metode imunohistokimia.

Hasil menunjukkan bahwa fraksi protein daun *C.papaya* mempunyai aktivitas sitotoksik pada sel kanker payudara (T47D) dengan harga IC_{50} 2,8 mg/mL. Fraksi protein tersebut mampu menaikkan ekspresi protein p53 sebesar 59,4% dan menurunkan ekspresi protein Bcl-2 sebesar 63%. Hasil ini mengindikasikan bahwa fraksi protein mampu menginduksi apoptosis.

Kata kunci: fraksi protein, *Carica papaya* L. , ekspresi protein p53 dan Bcl-2

Abstract

Carica papaya L. leave was known containing *Ribosome-inactivating proteins* (RIPs), which demonstrated to have an *in vitro* cytotoxic effect on cancer cell lines. This research examined the effect of protein fraction containing RIPs isolated from *Carica papaya* L. on the expressions of p53 and Bcl-2, regulator proteins on apoptosis process, in breast cancer cell lines (T47D).

RIPs from *Carica papaya* L. leaves were isolated by amonium sulfat precipitation. This fraction was analyzed by the activity of cleaving supercoiled double stranded DNA, in order to identify the presence of RIP. The active fraction was then tested of the citotoxic activity on breast cancer cell lines followed by analysing the expressions of p53 and bcl2 using immunohistochemistry technique.

The results indicated that the protein fraction possessed citotoxic activity in breast cancer cell line with the IC_{50} of 2.8 mg/mL. The expression level of p53 was increased by 59.4%, while Bcl-2 protein was decreased by 63%. These results suggested that this protein could induce apoptotic process.

Key words : protein fraction, *Carica papaya* L. , expression of p53 and Bcl-2

Introduction

Carica papaya L demonstrated to contain ribosome-inactivating protein (RIPs) as indicated by the ability of cleaving supercoiled DNA *in vitro* and N-glycosidase on rRNA yeast (Purnamawati, 1997, Sismindari dan Lord, 2000; Rumiayati *et al.*, 2000). The protein was purified by column chromatography and had the molecular weight about 32 kDa (Rumiayati *et al.* 2000). The protein was found to have a cytotoxic activity on HeLa cancer cell-lines (Rumiayati *et al.*, 2003).

Ribosome-inactivating proteins (RIPs) are protein toxins widely distributed throughout the plant kingdom, which are toxic to other organism. The toxic effects of RIP are attributed to the inhibition of protein synthesis by inactivating ribosomes through the cleaving of the N-glycosidic bond at the A₄₃₂₄ position of 28S RNA so that they are no longer able to function in protein synthesis (Barbieri *et al.*, 1993). Apart of the activity on ribosomal RNA, several RIPs demonstrate to exhibit a unique enzymatic activity on cleaving supercoiled double stranded DNA into the nicked circular and linear form. RIPs only act on supercoiled and nick-circular DNA and seldom cleave the linear form of the same molecule (Ling *et al.*, 1994). They also have cytotoxic activity that makes them an excellent candidate as the toxic part of immunotoxin for cancer therapy (Goldmacher *et al.*, 1994). The rRNA depurination activity of RIPs has role in its cytotoxic activity (Bagga *et al.*, 2002).

Many studies reported that RIPs were capable to kill cells by inducing apoptosis in a wide variety of cell lines (Narayanan *et al.*, 2005). Apoptotic mechanism involved in RIP-induced cell death has been proposed by some reports (Ikawati, *et al.*, 2003, Narayanan *et al.*, 2005). Ricin, a RIP type 2 isolated from *Ricinus communis*, was found to induce both the apoptotic morphological changes and activation of caspase-3. The other study revealed that RIPs caused the ribotoxic stress response through their RNA-N-glycosidase activity, resulting in the activation of SAPK/JNK and p38 MAPK leading to apoptosis (Narayanan, 2005). Abrin induced apoptosis in Jurkat cells via the mitochondrial pathway of apoptosis. The proposed modes of this pathway are a loss

in mitochondrial membrane potential (MMP), rapid release of cytochrome c, activation of caspase-9 and a marked increase in the production of reactive oxygen species (ROS) in cells. RIPs were also able to induce apoptosis by down regulation of anti-apoptotic factors. Shiga toxin (ST), RIP from bacteria, was able to decrease in the cellular level of the BCL-2 family (Narayanan *et al.*, 2005).

Breast cancer is one of the leading causes of cancer death in the world. It is caused by mutation of p53 gene, Breast Cancer Antigen-1 (BRCA-1) gene, Breast Cancer Antigen-2 (BRCA-2) gene and amplification of *c-Myc* (Miller *et al.*, 2005; Vita *et al.*, 2001).

Mechanism of compound as chemotherapeutic agents may pass through induction of apoptosis or anti-proliferation in cells. One of the targets of apoptotic induction in the mitochondrial pathway is a member of the Bcl-2 protein family. This member can regulate releasing of cytochrome c that is important to activate cascade of caspase to active executioner caspase. It can cleave the cellular substrates that lead to characteristic biochemical and morphological changes of cells. (Burlacu, 2003; Kuwana and Newmeyer, 2003). Chemotherapeutic agents are indicated that they can induce of the apoptotic program with the mechanism as follows : modulate oncogenes that can enhance the expression of tumor suppressor genes (p53), increase expression of Bax protein, a pro-apoptosis members, and decrease the expression of Bcl2, an anti-apoptosis members (Xu, *et al.*, 2001).

In this research, analysing the mechanism of protein fraction of *C. papaya* L. on the induction of apoptosis through the mitochondria pathway was carried out. The results demonstrated that, protein fraction of *C. papaya* L. was able to induce apoptotic process by increasing the level of p53 and decreasing of level of Bcl-2 protein.

Methodology

Fresh *Carica papaya* L. leaves were obtained from local garden at Yogyakarta. A voucher specimen was deposited in the Laboratory of Life Science Gadjah Mada University, Yogyakarta, Indonesia. DNA pUC18 was obtained from laboratory stock of Life Science Laboratory, LPPT GMU. Breast cancer cell-lines (T47D) were

obtained from Prof. Tatsuo Takeya (Nara Institute of Science and Technology Japan).

Preparation of protein fraction *Carica papaya* L leaves

C. papaya L. leaves extract was prepared by grinding the leaves in 5mM sodium phosphate buffer pH 7.2 containing 0.14 N NaCl (10 mL per g). The extract was precipitated by 100% saturated $(\text{NH}_4)_2\text{SO}_4$ with overnight stirring at 4°C and then centrifuged at 28000 g for 30 minutes. The precipitated protein was dialyzed in sodium phosphate pH 7.2 overnight at 4°C (Sismindari *et al.*, 1998).

Preparation of supercoiled DNA

Escherichia coli DH5 α harboring pUC18 was cultured in LB medium containing ampicillin 150 mg/mL at 37°C. After reaching the stationary growth phase, total plasmid DNA was purified by the modified alkaline lysis procedure (Eperon, 1989).

Cleavage of supercoiled DNA with RIPs

One μg of supercoiled double stranded plasmid DNA (pUC18) was incubated with various amounts of the protein fraction containing RIPs to a final volume of 20 μL in 50 mM Tris-HCl, 10 mM MgCl_2 , 100 mM NaCl, pH 8.0, at room temperature for 1 hour. At the end of the reaction, 10 μL of loading buffer (30% glycerol, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF) were added. Electrophoresis was carried out using a 1% agarose gel in 0.5 x TBE (tris-borat) buffer. DNA bands were visualized by staining the gel with ethidium bromide. Incubation of linear DNA (*Eco*RI-linearised pUC19) with protein fraction was carried out as described above.

Preparation of T47D cancer cells

Breast cancer cells-line, T47D, was grown in RPMI 1640 (Sigma) medium containing 5% v/v Fetal Bovine Serum (FBS) (Sigma) and 1% v/v fungison (Sigma) in the presence of 1% w/v of penicillin-streptomycin (Sigma) under 5% CO_2 at 37°C.

Cytotoxic activity of protein fraction of *Carica papaya* L.

The cytotoxic activity of protein fraction isolated from *C. papaya* L. was assayed on breast cancer, T47D, cell-lines. Adherent cells were plated in a 96-well plate at a density of 5×10^4 cells/well in 0.1 mL of RPMI medium containing 10% FBS, and incubated with various concentrations of protein fraction for 24 hours under 5% CO_2 at 37°C. Following incubation the cells were counted using

haemocytometer (Hanif *et al.*, 1997). Activity was plotted as percentages of cells survive compared to the control (untreated cells). The results were expressed in the form of IC_{50} values.

Assessment of apoptosis using Double Staining Methods

Breast cancer cells-line, T47D, (1×10^6) were treated with the protein fraction 0,451 mg/mL for 24 h at 37°C on plate with cover slips. Following incubation, cover slips were fixed on objective glass and were treated by Etidium Bromida-Acridine Orange. After 30 minutes incubation, morphology of the cell was analyzed under fluorescence microscope.

Identification of p53 and BCL-2 using Immunocytochemistry technique

The acetone-fixed cells were pretreated by micro waving in citrate buffer (10 mmol/L, pH 6.0) for 7 minutes at 600 W two times. The sections were then incubated with the monoclonal antibodies for p53 and BCL-2, DBA for 1 hour followed by biotinylated horse anti-mouse immunoglobulin as the secondary antibody and 3-amino-9-ethyl-carbazole (for DBA.44) as chromogens in the presence of H_2O_2 . The p53 and BCL-2 expression were identified by the present of dark brown color of cells.

Results and Discussion

Cleavage of double stranded DNA by protein fraction of *Carica papaya* L.

Activity test on cleaving supercoiled double stranded DNA (pUC18) was carried out in order to prove the presence of RIPs in the protein fraction of *Carica papaya* L. In this experiment, pUC18 was incubated with series concentration (2mg/mL – 10 mg/mL) of protein fraction at 25°C. It was demonstrated that, the supercoiled DNA band (a) in the agarose gel started gradually fainter at a concentration of 2mg/mL, while nicked (c) and linear bands (b) began to appear at the increasing concentration as indicated by slightly increasing the intensity of these bands (Figure 1). The supercoiled DNA completely disappeared at the concentration of 10 mg/mL. The disappearing of supercoiled DNA indicated the higher activity of the protein fraction. The results demonstrated that the protein fraction of *C. papaya* L. contained RIPs like protein.

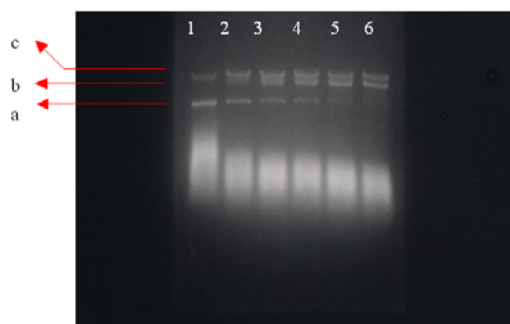


Figure 1. Cleaving activity of supercoiled pUC19 by protein fraction *Carica papaya* L. at series of concentration: (1): untreated pUC19; (2): 2 mg/mL, (3) : 4 mg/mL, (4): 6 mg/mL, (5): 8 mg/mL (6): 10 mg/mL. (a): supercoiled DNA, (b): linear DNA, (c): nicked circular DNA

Cytotoxicity activity of protein fraction in Breast cancer cells-line (T47D)

Breast cancer cell lines (T47D) was treated with various concentrations (0,08 – 5 mg/mL) of the protein fraction (Figure 2). At the concentration of 5 mg/mL, the protein fraction had a 55,83% inhibition whereas at the lowest concentration, it had 13,33% inhibition. The value of IC_{50} (a concentration at 50 % inhibition) that was analyzed by probit method was approximately 2,84 mg/mL. Based on the IC_{50} value, the protein fraction of *C.papaya* was less cytotoxic comparing to, basic (MJ-30) and acidic (MJ-C) RIP-like proteins isolated from *Mirabilis jalapa*, which had the IC_{50} of 361 μ g/mL and 27.8 μ g/mL respectively on the same cancer cell-line (Ikawati *et al.*, 2006; Sudjadi *et al.*, 2006). The differences of the cytotoxicity of these proteins might caused by the fact that protein fraction of *C.papaya* was unpurified protein, whereas MJ-30 and MJ-C were purified proteins using coloum chromatography. Further analysis, induction apoptosis and measuring the expression of p53 and Bcl2, were carried out to confirm the activity.

Effect of the protein fraction on the induction of apoptosis

Induction of apoptosis was carried out by incubating the T47D cell-line with the protein fraction at the concentration of 0,451 mg/mL and double stained with mixed

ethidium bromide-acrydine orange. The result demonstrated that the protein fraction was able to induce late apoptosis, as indicated by the appearance of apoptotic body with the orange color of the treated cell (Figure 3B). Whereas, at the untreated cell, orange color was not observed (Figure 3A). Therefore it was strongly suggested that the protein fraction of *Carica papaya* L. induced the apoptotic processes in breast cancer cells-line. The induction of apoptosis was also demonstrated by MJ-30, RIPs-like protein isolated from *Mirabilis jalapa* on cervical cancer cells, HeLa cells-line, (Ikawati *et al.*, 2003).

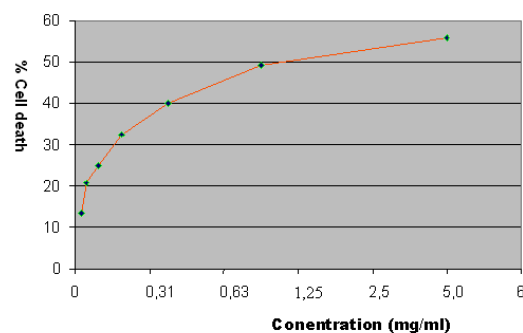


Figure 2. Inhibition of protein fraction *Carica papaya* in breast cancer cells-line at a series concentrations of 0,08–5 mg/mL.

The mechanism of apoptotic in breast cancer cell lines was proposed due to the RNA-N-glycosidase activity of the protein fraction. RNA-N-glycosidase activity led to damage of 28S rRNA resulting in an activation of SAPK/JNK thus activating a ribotoxic stress response and apoptosis (Narayanan, 2005). Induction of apoptosis was also proposed via mitochondrial pathway involving the regulation of tumor suppressor gene (p53) expression as well as an anti-apoptotic factor (Bcl-2 protein).

Analysis of p53 and Bcl-2 proteins expressions using immunocytochemistry method

As shown in Figure 4, the expression of p53 in breast cancer cells treated with protein fraction (B) was significantly increased at about 59,4% compared to the untreated cells with protein fraction (A). On the other hand, the expression of Bcl-2 protein, the anti-apoptotic protein, in breast cancer cells-line treated with

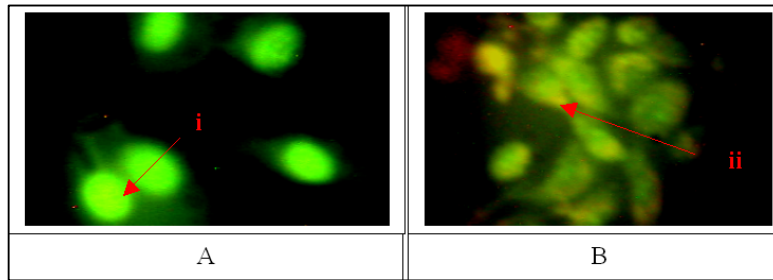


Figure 3. Image of stained breast cancer cells-line with mixed dye Ethidium bromide-acridine orange. (A) untreated cells (B) treated cells with protein fraction of *C.papaya* (i) viable cells (ii) cells with late apoptosis

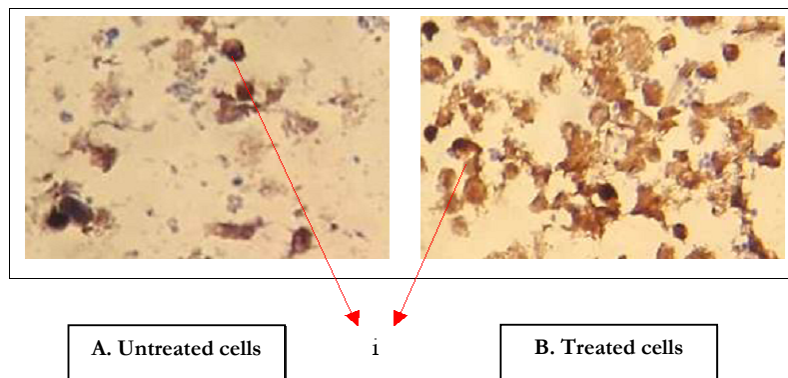


Figure 4. Analysis of expression of Bcl-2 protein on breast cancer cells-line using immunocytohemistry method. (A) Untreated Cells (B) treated cells with protein fraction of *C.papaya* (i) Cells expressed p53 protein. There was increase in expression of p53 protein level in treated cells.

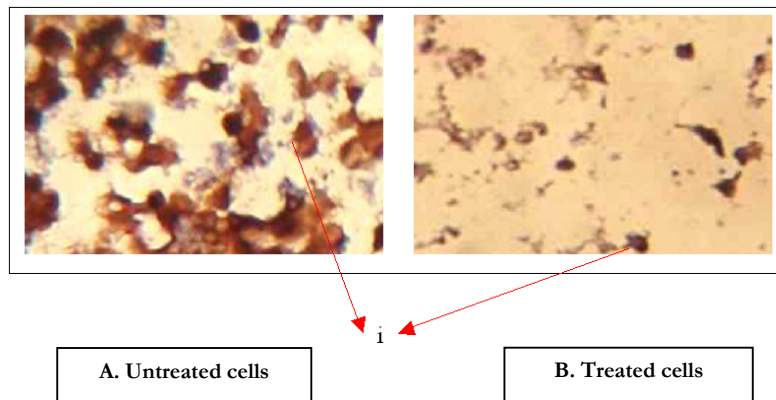


Figure 5. Analysis of expressions of Bcl-2 protein on breast cancer cells-line using imunocytohemistry method. (A) Untreated cells (B) treated cells with protein fraction of *C.papaya*. (i) Cells expressed Bcl-2 protein. There was decrease in the expression of Bcl-2 protein level in treated cells.

protein fraction (Figure 5 A) was decreased at about 63% compared to the untreated one (B).

These results suggested that the mechanism of apoptosis induced by the protein fraction in breast cancer cells-line was regulated via mitochondrial pathway and passed through p53-dependent. The protein fraction of *C. papaya* L. influenced the expression of p53 protein and its downstream process, Bcl-2 protein. However, the mechanisms by which protein fraction increased the expression of mutated p53 protein in breast cancer cells-line need to be investigated further.

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Conclusion

The protein fraction possessed cytotoxicity activity in breast cancer cell lines with IC₅₀ 2,843 mg/mL. The protein fraction can induce apoptotic processes in breast cell-lines. The level of p53 protein in breast cancer cell-lines was increased by 59,4%, while Bcl-2 protein was decreased by 63%.

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